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# THE FUNCTION OF CREATINE IN MUSCULAR CONTRACTION.

by

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*(Submitted for publication 6th February, 1925.)*

## INTRODUCTORY.

It was in the investigations of Fick, carried out over fifty years ago, that the idea arose of two distinct chemical processes underlying the contraction and relaxation phases of a single muscle twitch. This conception, disregarded at the time, has during recent years been clearly established by the researches of Fletcher and Hopkins, of A. V. Hill, and of Meyerhof; and it is now quite certain that while contraction is caused by the liberation of lactic acid on to certain sensitive surfaces within the muscle fibres, relaxation occurs when this acid is again removed, not, indeed, by oxidation, as was for a time believed, but simply through neutralization by one of the basic substances contained in muscle.

The work of Meyerhof (20) has made it appear probable that it is the muscle proteins that bring about this neutralization, the sodium salts of protein reacting with lactic acid to form sodium lactate and the very weakly dissociated acid-protein. The investigation has necessarily turned mainly about one point, namely, an attempt to correlate the heat of formation and neutralization of a given quantity of lactic acid in muscle, with the heat of formation and subsequent neutralization by protein buffers, of the same quantity of acid, when carried out in laboratory glassware. The formation of 1 gr. lactic acid in muscle is accompanied by the liberation of 370-410 calories (19, 21), average about 389 calories (21). Combustion experiments on glycogen and lactic acid reveal a value of 188 cal. as the heat of formation of lactic acid from glycogen. During the neutralization of 1 gr. lactic acid by protein buffers of muscle about 140 cal. are produced. In his most recent work (21) Meyerhof believes that the remaining 60-70 calories are identical with the delayed anaerobic recovery heat of Hartree and Hill (12), and that dehydration of protein in a non-aquaeous phase underlies this recovery heat, and also the unexplained 60 to 70 calories.

My own studies on the structure of muscle fibres have made it appear more probable that the neutralizing base is formed simultaneously with the lactic acid—

the acid on the surface of the one excitable membrane, the base on that on the other (31). It would be difficult to conceive of a protein, devoid of basic properties, which could, moreover, be rendered basic by a simple chemical change; and this would indicate that muscle protein was not the base concerned.

The readiness with which we accept this view depends upon the interpretation we place upon the histological observations. If we regard the structure that the microscope reveals as the actual contractile machine, then the conclusion I have reached appears quite unavoidable. The sarcomeres are regarded as the contractile units of the fibres; the two membranes of Krause are considered to be the transmitters of the excitation; and the interfibrillar spaces are the regions in which the chemical changes, which cause contraction, take place. On this interpretation, we regard transmission of stimuli as the property of two well-defined membranes, and not a property of muscle protoplasm in general. The organization of the fibre, moreover, is such that the stimulus must necessarily come into close relation with all the individual contractile elements. This theory, however, encounters the difficulty that it is incompatible with the view of a preformed base, ready in quantity sufficient to neutralize the lactic acid liberated at every twitch. Our confidence is, however, restored when we examine the one case in which the animal is able to dissociate the contraction and relaxation phases of a single twitch, namely, the claw muscle of the crayfish. Here each muscle fibre is provided with two nerves, and one nerve terminates upon one of the excitable membranes, the other on the second. Evidently, then, one of the excitable membranes is concerned with contraction, the other with relaxation. The mechanical phenomena of contraction, as determined experimentally, can be deduced quite readily from this conception (31).

Those who look upon contraction as the property of certain ultramicroscopic colloidal complexes would see in the microscopic structure of a muscle fibre merely a scaffolding, whose function it is to form a support for a contractile mechanism, which we can never hope to see. This physico-chemical interpretation of muscle action has so far met with only very limited success. It considers contraction to be due to an increase in the convexity of ovoid colloidal particles, the change being produced by an increase of their surface tension under the influence of lactic acid liberated. It is able to offer an explanation of the delayed relaxation that occurs in fatigued muscle—as being due merely to the decreased alkalinity of the muscle as the buffer substances become exhausted, acting of course on the "mass action" principle. (The validity of this explanation is, however, doubtful, for it seems to confuse fatigue with rigor.) But as regards the interpretation of the more detailed features of muscle contraction, such as the form of the twitch curves, it has hitherto proved of very little help. Moreover, in a recent paper (6), W. O. Fenn, comparing the heat production of muscles doing work with that of muscles actively contracting, but not doing work, finds that in the former

case the heat production is very considerably greater than in the latter; he compares the contractile mechanism with an electric motor, in which the energy used increases with the work done, and concludes that "the necessity of providing a muscle with a mechanism of this general type makes demands upon theories of muscular contraction which at present neither the surface tension nor the water absorption theories seem able to meet". I have tried to show, on the other hand (32), that this excess heat follows as a direct consequence of the nature of the contractile machine that the microscope reveals. There is one further case in which the physico-chemical theory in its present position seems inadequate, namely, in its incapacity to explain tonus. Tonus will eventually probably be found to be due to an increased acidity of the muscles, due, possibly, to stimulation of one only of the excitable membranes. How could such a phenomenon occur if the lactic acid could be neutralized without any further hindrance? Not only does this physico-chemical theory, which takes no heed of the visible structure of the fibres, fail to explain most of the phenomena of muscular action, but histological observation contradicts it directly. When we compare under the microscope the appearance of contracted and relaxed sarcomeres, we find that the contracted ones are much shorter and thicker than those in relaxation. It might be urged that this is due to the contraction of the ultramicroscopic colloidal complexes contained within these sarcomeres. But this is not so, for the contraction of the sarcomere is quite clearly due to the movement of fluid within the sarcomeres, easily visible under the microscope. A darkly staining fluid retreats from the doubly refracting region in the middle of each sarcomere and accumulates at its two ends. It causes them to thicken, and unless the substance of the sarcomere stretches, it must shorten. This movement of the fluid contents of the sarcomeres is very clearly observed when we examine the successive "striations" in fixed contractile waves; and the fluid movements are so clearly seen to be confined to the contents of the sarcomeres that it seems remarkable that the less obvious interpretation of movement of interfibrillar fluids should ever have been placed upon it. Failure to recognize the meaning of the doubly refracting material doubtless explains the confusion that has prevailed.

The main difficulty which the theory that I have put forward has encountered is that it postulates the liberation of a basic substance during activity, simultaneously with and in equivalent quantities to the acid liberated. The suggestion was made to me at the time by Professor Brailsford Robertson, that creatine, of whose function nothing was known, and which has been found to increase in quantity during tonus (26), might be the substance concerned. A subsequent examination of the scattered literature on the subject revealed a number of highly suggestive observations, pointing to the conclusion that creatine was, in fact, the substance which causes muscular relaxation.

*The Behaviour of Creatine in Muscles during Muscular Activity.*

Creatine is a rather inert and very feebly basic substance. It occurs in the animal body mainly, but not entirely, in the muscles; and its content in the different muscles is roughly proportional to their activity; it is present in largest quantity in the skeletal muscles, especially in the quickly contracting "pale" muscles; the creatine content of cardiac muscle is considerably less, while in the sluggish plain muscle it is present in only very small quantities. It occurs in much larger quantity in the pregnant than in the non-pregnant uterus (2).

Observations on the creatine content of resting and fatigued muscle have given rise to a number of apparently contradictory statements. Liebig found a marked increase in the creatine content accompanying fatigue. Monari (22), employing the zinc chloride isolation method, found a doubtful increase of creatine, but a very pronounced increase of creatinine, accompanying muscular fatigue. Other workers have recorded an increase of muscle creatine (Sarokow, Sczelkow), while Nawrocki, and also Voit, record a decrease.

The quantitative isolation of creatinine is an almost impossible task, and much greater reliance must be placed on the recent investigations in which the Folin colorimetric method has been employed. Using this method, E. Mellanby (18) failed to observe any increase in the creatine content of fatigued frog's muscle, deprived of its circulation. He also failed to observe any creatinine formation during activity of the muscles of an anaesthetized rabbit, with circulation intact. Brown and Cathcart (3, 4), on the contrary, report an increase of 6–13% in the total creatine-creatinine content of a frog's nerve muscle preparation, after stimulation; while in the decerebrate frog, with circulation intact, there is a loss of 6–15%. W. H. Thompson, using decerebrate cats, could find no loss of creatine during intermittent stimulation, with circulation intact, but observed a loss of 6% with restricted circulation (30). Finally, Brown and Cathcart (4), using anaesthetized rabbits with circulation intact, find, after strong stimulation for about half an hour, a decrease of "total creatinine" of from 10 to 22 %, while in two experiments in which the animal was allowed to breathe, not air, but pure oxygen, the loss of creatine was within, or only just outside, the experimental error.

These apparently contradictory results all point in one direction, namely, that there is a creatine precursor in muscle, that during stimulation creatine is converted into a diffusible substance which on boiling with acids is converted into creatinine, and that oxygenation reconverts this diffusible substance into creatine again. The existence of a creatine precursor in muscle is quite definitely proved by the observation of Pekelharing and van Hoogenhuyze (26) that isolated frog muscle during tonus and post-mortem rigor undergoes a very marked creatine increase. The observation of Weber (35) and of Howell and Duke (15) must also be mentioned, namely, that the beating heart liberates a small quantity of creatine into the perfusing fluid.

The view that creatine had any so important a function as to produce muscular relaxation appeared to be contradicted by the report of E. Mellanby (18) that creatine does not develop in the embryonic chick till the twelfth day, i.e., nine days after the heart begins to beat. I have, however, been able to detect creatine in very much younger chick embryos (33). It is perhaps necessary to remark that a very good quality of picric acid is necessary to detect minute quantities of creatinine.

## EXPERIMENTAL.

The experimental work was directed towards trying to determine the formation, during prolonged excitation of muscle, of a substance with basic properties, which could arise from creatine by a fairly simple chemical change. The hind limbs of frogs, mainly *Limnodynastes*, occasionally *Hyla*, were used throughout.

### (1) *The Possibility of Creatinine as the Base.*

The occurrence of creatinine in the urine, the fact that it is a considerably stronger base than creatine, and the statement of Monari (22) that there was a great increase in the creatinine content of muscles undergoing fatigue, suggested that creatinine might be undergoing a simple transformation into its anhydride during excitation, and that this might be the basic substance concerned. It was somewhat a surprise to find that this view had already been stressed by Ranke in 1865. He considers not only lactic acid, but also creatine, to be a fatigue product of muscle, and believes that the fatigue products can be removed from muscle by the blood and lymph, the lactic acid being neutralized by the alkaline substances present in muscle. Since, however, muscle devoid of circulation can undergo recovery from fatigue, he concludes that "while a part of the creatine is converted into creatinine, the newly formed creatinine serves, in virtue of its basic properties, to neutralize the acid" (27).

E. Mellanby (18), however, using the colorimetric method, failed to detect any creatinine formation in muscles as fatigue progressed. I can confirm his conclusion.

### (2) *The Possibility of the Transformation of Creatine into Methylguanidine or Dimethylguanidine.*

The strongly basic properties of these substances, their reported occurrence in muscle, and in normal human urine, and their reported increase there during abnormal muscular activity (25), suggested that creatine might be undergoing a change into one of these two substances. At the same time, there were a number of serious objections to this view. These substances do not give the Jaffe test on heating in acid solutions, so that the increase of total "creatinine" observed by Brown and Cathcart could not be accounted for. Moreover, if these substances

once found their way into the circulation they would give rise to very serious symptoms (25), and yet one might readily expect that the fatigue products during heavy work should leak out to a considerable extent into the blood. Furthermore, the transformation of creatine into one or the other of these two substances would involve the liberation either of an equivalent quantity of acetic acid, or of carbon dioxide, respectively. Fletcher (7) has found, however, that the carbon dioxide output of a muscle contracting anaerobically is independent of the actual work it is doing, while if acetic acid were being liberated from the creatine during the formation of methylguanidine, then the methylguanidine would necessarily have to act as a diacid base.

Nevertheless, the isolation of methylguanidine from muscle by a number of workers rendered this hypothesis so probable that much time was spent in trying to demonstrate the formation of this substance during fatigue. While working on these lines, I had the good fortune to discover the colour reaction of free guanidine bases with "sodium nitroprusside", which I have previously reported (33), and the results obtained by this reaction finally disposed of the view that free guanidine base could be the substance sought.

(3) *The Possibility of the Transformation of Creatine into a more Basic Isomer of Creatine.*

Bearing in mind the recent work of E. A. Werner (36) on the structure of urea, and the fact also that creatine, if the structure we usually assign to it were correct, should be a fairly strongly basic substance, it occurred to me that the inertness of creatine and its very feebly basic properties might be due to a cyclic arrangement of the guanidine portion of the molecule, and that this substance might become much more strongly basic by the opening of this ring. The substance so formed might readily be converted into creatinine on heating with acid, and so could be included in our "total-creatinine" estimations of muscle. The failure to detect any decrease in the creatine content of isolated muscle undergoing fatigue would, therefore, find a ready explanation. The slight increase which is actually observed might be interpreted as representing the formation of creatine from some precursor in muscle, perhaps arginine or lecithine (in analogy with the replacement of glucose from glycogen as the former was being transformed into lactic acid).

The method used in studying lactic acid formation in muscle could not be employed, since we would have no method of distinguishing this creatine isomer from creatine itself. It was, therefore, planned to perfuse the muscle with Ringer's fluid, and examine this fluid for its fatigue products. On account of the small size of the animals this was found to be impracticable, and the method of simply suspending the limbs in Ringer solution had to be resorted to. For a reason that will be considered later, this seems to be the only method of investigating the problem, and perfusion experiments would probably have yielded data that would have been difficult to interpret.

EXPERIMENT 1. *The Liberation of "Creatine" from Fatigued Muscle.*

Four frogs (*Limnodynastes*) were placed in a receptacle of ice-cold water for about thirty minutes till thoroughly numb; they were then "pithed", the hind legs separated from the body, and skinned as carefully as possible. Two pairs, serving as controls, were suspended for 2¼ hours at room temperature (21°C) in 20 cc. buffered Ringer solution. The other two were subjected to strong direct stimulation from an induction soil for about thirty minutes till thoroughly fatigued. During the stimulation one pair lay in Ringer's fluid, which was subsequently rejected; the other was stimulated on moist cotton wool. The fatigued limbs were then suspended for 2¼ hours in buffered Ringer, similarly to the controls. Subsequently one of the controls, which had undergone but little fatigue while suspended, was stimulated, on moist cotton wool, till fatigued, and then suspended once again for 2¼ hours in Ringer's fluid.

The Ringer solution, which should now contain the fatigue products that had diffused into it from the muscle, was examined quantitatively for creatine (creatinine was absent). The creatine estimation was carried out in the usual way: 20 cc. fluid, acidified with 1 cc. strong hydrochloric acid, was autoclaved for 20 minutes, at 117°C. On cooling 15 cc. concentrated picric acid was added, then 10 cc. of 10% sodium hydroxide. After standing four minutes this was made up to 100 cc. and estimated colorimetrically against a known strength of creatinine zinc chloride. The following results were obtained:—

| Exp. | Treatment. | Weight. | "Creatine" Liberated* | Remarks. |
|---|---|---|---|---|
| A. | Unstimulated Muscle | 5·5 gr. | ·46 mg. | Muscles considerably fatigued after removal from Ringer fluid. |
| B. | Unstimulated Muscle | 7·5 gr. | ·31 mg. | Muscles had not undergone much fatigue while suspended. Used again in Exp. E. |
| C. | Stimulated to Fatigue | 6·9 gr. | 1·83 mg. | Muscles in rigor after removal from Ringer fluid. |
| D. | Stimulated to Fatigue | 6·8 gr. | 2·26 mg. | Muscles in rigor after removal from Ringer fluid. |
| E. | Stimulated to Fatigue (Same muscle as in Exp. B.) | 7·5 gr. | 4·34 mg. | Muscles in rigor after removal from Ringer fluid. |

* In all cases "creatine" has been calculated as creatinine.

From this experiment it is seen that fatigued muscles liberate from four to fourteen times as much "creatine" into the fluid in which they are bathed as do muscles that have previously not been fatigued. It seems probable that the "creatine" liberated by unstimulated muscles depends upon the amount of fatigue they undergo while suspended. It is well known, of course, that lactic acid accumulates under similar conditions; for example, while the "lactic acid maximum" of frog's muscle is about ·2%, Meyerhof finds a resting lactic acid production of ·02% per hour (19).

The experiments of Brown and Catheart have shown that there has occurred only a slight increase of "total creatinine" in muscles which have undergone fatigue. It is clear, therefore, that the creatine has been rendered very much more diffusible as the result of excitation.

EXPERIMENT 2. *The Effect of Oxygen upon the "Creatine" of Fatigued Muscle.*

Three limb pairs of frogs (*Limnodynastes*) were taken, and, with the skin still present, stimulated to fatigue. They were then treated as follows:

A. Skinned and placed on moist cotton wool in a continuous stream of oxygen for five hours.

B. Skinned and placed in a vessel containing about 90% stationary oxygen.

C. As in B, but skin not removed.

After removal from the oxygen C. was skinned, and the three limb pairs were suspended for 1½ hours each in 20 cc. Ringer's fluid. Weight of each limb pair about 5½ grams.

The "creatine" content of the fluid was then estimated. The results were as follows:

| | "Creatine" Liberated. |
|---|---|
| A. - - - - - - | ·76 mg. |
| B. - - - - - - | 2·85 mg. |
| C. - - - - - - | 4·11 mg. |

From this it appears that the diffusible "creatine" has to a large extent been converted back into the non-diffusible form. In Experiment A the oxygenation of the outer portions of the muscles may be regarded as fairly efficient. In B this was certainly not the case; during the first fifteen minutes oxygenation was probably satisfactory, but it was found quite impossible to keep the muscle properly moist longer than this. In Experiment C the muscle was under fairly anaerobic conditions (it is believed, however, that frogs can absorb some oxygen through their skin).

However striking the difference in the "creatine" output of these three muscles may be, yet when we compare the loss of creatine of the well oxygenated muscle with that of the resting muscle of the previous experiment (in which two

muscles, slightly heavier and exposed for an hour longer, lost only ·46 and ·31 mg. respectively), we find that the muscle creatine cannot have been restored entirely to its resting condition. The following explanation may be offered for this. During the five hours' exposure to oxygen the muscle is constantly undergoing fatigue; the oxygen would bring about an extensive recovery in the outer portion of the muscle, but owing to the small solubility of oxygen would probably get little opportunity to penetrate into the deeper parts. Hence, when this muscle is suspended in Ringer solution, the traces of fatigue products in the outer region, as well as some of that from the deeper portions, where it must be present in much larger quantities, will diffuse outwards.

This same difficulty was encountered by Fletcher and Hopkins in their work on lactic acid in amphibian muscle (p. 285). When the entire limbs of frogs were used, the lactic acid could not be reduced below ·1%, i.e., to about half the lactic acid value of fatigue; whereas, when a chain of fatigued gastrocnemii were immersed in oxygen, lactic acid appeared to be entirely removed (8).

In order to bring about a more marked difference between the "creatine" output of muscles which had been fatigued and those which had undergone oxygenation subsequently to fatigue, the following facts were considered: Fletcher and Hopkins had found that the persistence of irritability of muscle was necessary if oxidative removal of lactic acid should take place. Since frog's muscles stimulated directly to fatigue entirely lose their irritability and subsequently pass into rigor under anaerobic conditions, it was considered advisable not to stimulate the muscle beyond end-plate fatigue. Secondly, it was thought advisable to reduce the period of oxygenation, since this would diminish the subsequent formation of fatigue products in the deeper parts of the muscle, while the maximum effects of oxygenation would already, as Fletcher and Hopkins have shown, have occurred. Finally, the muscles were to be exposed for only one hour to Ringer's solution; this, it was hoped, would diminish the diffusion of fatigue products from the deeper parts of the muscle. The data so obtained would, of course, only be comparable among themselves, and no comparison could be drawn with the results obtained from muscles that had been much longer exposed.

The experiment was carried out as follows: Two hind limb pairs were stimulated to end-plate fatigue. One pair was then placed for four hours on moist cotton wool, in a continual stream of oxygen; the other was placed for a similar period on moist cotton wool in a corked test-tube containing air. At the end of the four hours the two limb pairs were removed and suspended for one hour in about 20 cc. Ringer solution; at the end of the hour they were removed and placed into another 20 cc. of Ringer fluid. The muscle which had been exposed to air was quite inexcitable, and was now rejected. The other was already markedly fatigued; it was stimulated to absolute fatigue, and then suspended

once again for one hour in 20 cc. Ringer fluid. A creatine estimation of the five fluids was then made. Results:

| | Weight of Muscle. | "Creatine" liberated during first hour's exposure. | "Creatine" liberated during second hour's exposure. | "Creatine" liberated after stimulating to fatigue. |
|---|---|---|---|---|
| A. Oxygenated after fatigue . . . . . | 6 gr. | ·7 mg. | ·55 mg. | 2·3 mg. |
| B. Not oxygenated after fatigue . . . . . | 7·2 gr. | 2·95 mg. | 2·1 mg. | Inexcitable |

While this experiment illustrates the effect of oxygenation on the removal of "diffusible creatine", it has failed to bring the "creatine" back to its resting condition, probably for the reasons already discussed.

EXPERIMENT 3. *The Relation between the Quantity of Lactic Acid and "Creatine" Liberated.*

The hind limb pairs of two frogs were stimulated, with skin still present, till fatigued; the skin was then carefully removed, the legs rapidly washed in Ringer solution, and then suspended in 22 cc. Ringer. After 1½ hours' exposure the legs were removed, and the lactic acid and "creatine" content of the fluid estimated.

For the estimation of the lactic acid, 10 cc. of solution were taken, traces of protein precipitated with tungstic acid, as recommended by Folin and Wu, and the solution then examined quantitatively for lactic acid by the bisulphite titration method of Clausen (5). For such complex mixtures as blood this method is liable to give results 50% too high (17), due mainly to the oxidation of α-hydroxy acids; but with the very simple solutions dealt with in this investigation it could probably be used with safety. It was found in practice that the procedure for the removal of glucose was superfluous, and it was discarded.

The following results were obtained:

A. *Limnodynastes.* Fatigued, and then placed for 1½ hours in 22 cc. Ringer's Fluid.

| "Creatine" liberated. (Calculated as Creatinine.) | Lactic Acid liberated. | Lactic Acid equivalent of "Creatine" liberated (calculated). |
|---|---|---|
| 3·26 mg. | 2·43 mg. | 2·59 mg. |

B. *Hyla aurea.* Treated as in previous experiment.

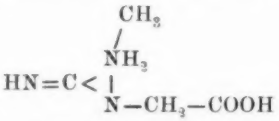
| "Creatine" liberated. | Lactic Acid liberated. | Lactic Acid equivalent of "Creatine" liberated (calculated). |
|---|---|---|
| 2·88 mg. | 2·37 mg. | 2·29 mg. |

From this experiment it appears that the lactic acid and the "creatine" are liberated in chemically equivalent quantities.

## DISCUSSION.

From the above experiments the conclusion must be drawn that during muscular activity the muscle creatine is undergoing a metabolism similar to that undergone by carbohydrate; the relatively inert glycogen and creatine are being transformed in equivalent quantities into much more active substances, which are capable of diffusing, as fatigue products, into the surrounding fluids. If the muscle is adequately oxygenated, these two fatigue bodies are restored, evidently, to their precursors (for the creatine content of muscle does not change). The question arises then as to the nature of the basic isomer into which creatine has been converted.

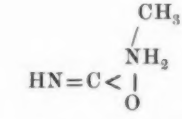
The recent work of E. A. Werner on the structure of urea (36) has cast a doubt on the accepted views concerning the structure of all substances containing the guanidine grouping. In the light of this work, I tested creatine for the free amino group that it is believed to possess, and find that it is absent. The formol titration method of Sörensen was employed. To a solution of 44 mg. pure creatine prepared from Liebig Meat Extract, 5 cc. formalin, just made neutral to phenolphthalein, were added. No detectable reduction of alkalinity occurred. From this it would appear that the structure we usually assign to creatine is not correct, and that, taking urea and methyl-urea as analogy, the correct formula should be:



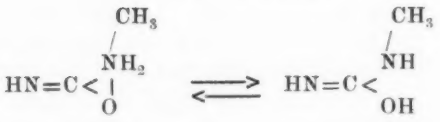
That creatine is methylguanidine acetic acid follows, of course, from its decomposition with barium hydroxide into urea and sarcosine. This cyclic formula for creatine would further explain its extremely weak basic properties; it must always have appeared rather an anomaly that creatine, possessing an amino group, should be a much weaker base than creatinine, in which the free amino group does not occur.

It seems to me very probable that the isomerism of the methyl-derivatives of urea offers the key to the problem.

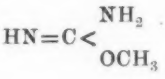
Methylurea



is a weak base; and in solution exists, it is believed, in equilibrium with iso-methylurea,



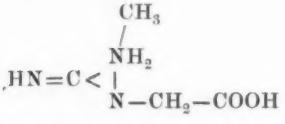
It is the "iso" form to which the weak basic properties are due, in the same way as the very feebly basic properties of cyclic urea in solution depend upon the isourea with which it is in equilibrium (36). On the other hand, "methyl-isourea,"



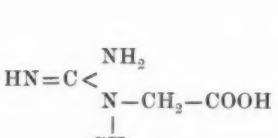
is a strong base.

The main point we notice about these substances is that the cyclic ureas open by the movement of a hydrogen atom, while the methyl group shows no tendency to move.

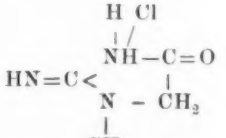
Now, in analogy with "methyl-isourea," we should expect creatine, as usually written, to be a strong base, this basicity being, however, somewhat diminished by the presence of the free carboxyl group; we can also understand why creatinine should be so much stronger a base than creatine. The three substances would be related as follows:



Creatine

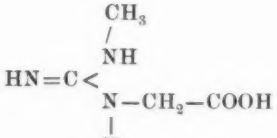


Basic Creatine



Creatinine hydrochloride

while creatine, when in the form of a salt such as the hydrochloride would be in the iso-form

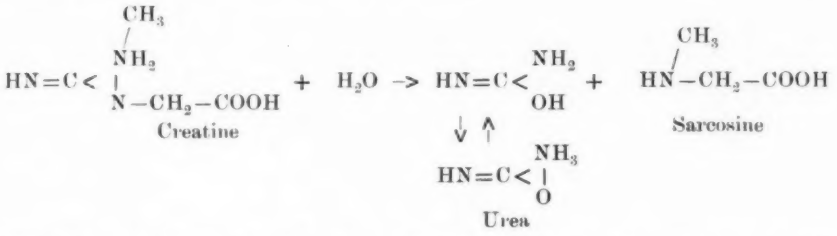


Iso-creatine

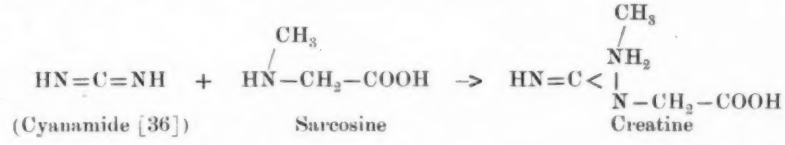
which, in analogy with urea and methylurea would be very strongly hydrolyzed and a feeble base.

I would suggest, therefore, that cyclic creatine is converted during excitation into basic creatine; that this substance, formed on one of the excitable membranes, neutralizes the lactic acid formed on the other, and thereby brings about relaxation of the muscle. During prolonged activity this substance accumulates, but during oxidative recovery is converted back into cyclic creatine, the energy necessary for the conversion being derived, of course, from the combustion of a certain part of the lactic acid (Meyerhof).

As an analogy for such a change, the decomposition, by barium hydroxide, of creatine into urea and sarcosine may be cited:




Here the methyl group clearly must move from the one nitrogen atom to the other, while the reverse movement of the methyl group is seen during the synthesis of creatine from cyanamide and sarcosine:




## THE BEHAVIOUR OF MUSCULAR CREATINE DURING EXERCISE.

When a muscle is undergoing easy intermittent activity within the body, then, provided the supply of oxygen is adequate, the oxidative recovery process is very efficient and relatively little of the fatigue products are able to diffuse from the muscles into the blood stream. But during very strenuous exercise this is not the case; the fatigue products accumulate in the muscles till the animal is exhausted, and it is only subsequently that their oxidative removal occurs (13).

Under these circumstances, the fatigue products will diffuse to a much larger extent that usual into the blood stream; and the question arises what will be the relative proportion of lactic acid and basic creatine liberated into the blood under these conditions? That the two substances might not necessarily be liberated in equivalent proportions follows from the following consideration: During severe physical exertion the muscles are strongly acid (otherwise they could not contract); an equivalent quantity of unneutralized basic creatine is present; there

is also the rapidly accumulating "basic creatine" lactate. These three substances will have unequal diffusion rates. The acid, as I have previously supposed, would have a greater diffusion rate than the base (34), but would be present in far less quantity than the neutralized acid.

Hence, while a muscle is undergoing active contraction with the circulation intact, one would expect considerably more lactic acid to diffuse from the muscles than creatine. It is for this reason that I believe perfusion experiments would not be likely to demonstrate the equivalence of lactic acid and basic creatine formation above described. The following experiment serves to illustrate this point:

EXPERIMENT 4. *The Relative Rates of Diffusion of Lactic Acid and Basic Creatine.*

The frog limbs, after removal of skin, were placed in a dish containing 40 cc. Ringer fluid, and stimulated for 35 minutes to fatigue. They were then removed, and placed for periods of 35 minutes into successive lots of 30 cc. Ringer fluid. In order to follow the course of the diffusion, the period of exposure had necessarily to be made rather short; however, a large amount of fatigue product was ensured by employing not one, but five limb pairs. The following results were obtained:

| | First 35 minutes. (Stimulation carried out in this fluid.) | Second 35 minutes. | Third 35 minutes. | Fourth 35 minutes. | Total. |
|---|---|---|---|---|---|
| Lactic Acid . . . . | 3·33 mg. | 2·19 mg. | 1·83 mg. | 1·17 mg. | 8·52 mg. |
| Creatine (calculated as creatinine) . . | 1·58 mg. | 2·85 mg. | 4·5 mg. | 4·7 mg. | 13·63 mg. |

The fluid in which the limbs were stimulated contains lactic acid in great excess over creatine; in the second fluid, the two substances are present in approximately equivalent proportions; in the next, the creatine predominates; this is even more marked during the last exposure. The experiment does not enable us to determine the true relative diffusion rates of the creatine and the lactic acid, since we cannot say how much of the creatine diffused out, in the first 35 minutes, in combination with the acid, and how much came out as free base. It is, however, quite clear that during the stimulation a large quantity of free acid must have left the muscles.

It should further be noted that the total lactic acid liberated (8·52 mg.) is considerably less than the calculated equivalent of the creatine liberated, namely, 10·8 mg. If the experiment had been continued longer the difference would doubtless have been more marked still. The experiment was, however, discon-

tinued because rigor had already set in.[1] This observation, indeed, seems to throw some light on the nature of rigor, and possibly also fatigue. We might assume that the basic creatine lactate compound undergoes a certain amount of hydrolysis; the quantity of resulting free acid and also free basic creatine might diminish the irritability of the excitable membranes, and the muscle would respond less and less to stimulation. On the other hand, rigor appears to be due to a retention of the free acid; this penetrating, from the interfibrillar spaces, into the sarcomeres and attacking these; being bound there by the protein buffers, and eventually bringing about a disorganization from which the tissue cannot any longer be rescued, even by exposure to pure oxygen.

This experiment also throws some light on the observations of A. V. Hill and Lupton (13) and of others on "oxygen debt"; following shortly upon very strenuous physical exertion, the respiratory quotient rises far above unity, and may acquire a value as high as 2·6. These investigators interpret their results in terms of the protein buffer neutralization theory, and conclude that as the muscles approach exhaustion the buffers become used up, and that the lactic acid must now diffuse into the blood; here the acid attacks the bicarbonates of the blood, carbon dioxide is turned out, and the respiratory quotient rises. In agreement with this is the observation of Ryffel (29) that following upon severe physical exertion large quantities of lactic acid appear in the urine. Doubt may, however, be cast upon the validity of this explanation. Diffusion of free lactic acid into the blood postulates a moderate lactic acid concentration gradient between the muscle fibre and the blood. If free acid is already present within the muscle, then, since the removal of this acid by diffusion must be a relatively slow process in comparison with the explosive rapidity of the acid formation at excitation, it would appear that the muscle, under these conditions, should pass into a condition of absolute rigidity. Such is certainly not the case. Any stiffness that follows upon strenuous activity is to be looked upon, more probably, as a purely reflex phenomenon. The great rise in the respiratory quotient appears rather to be due to the liberation of considerable quantities of free acid, due to the increased acidity of the muscles *throughout the whole duration of the exertion.*

Since an animal which is not doing severe work must be to some extent constantly forming fatigue products, it follows that these must be constantly passing in small amount into the blood. Even when the muscles are absolutely at rest this would appear to be the case. Frank and Voit have even reported a marked metabolism in muscles in the intact animal, after all excitation had been cut off with curare (10). (Compare the resting lactic acid formation of isolated

1 It will be noticed that if the creatine and lactic acid liberated during the first three exposures (i.e., before rigor had set in) be compared, the two substances are present in approximately equivalent proportion—8·93 mg. creatinine and 7·35 lactic acid (calculated lactic acid equivalent of creatinine = 7·11 mg.).

muscle.) This continued activity of muscle, even in the resting condition, appears to throw some light on the origin of urinary creatinine.

When considerable quantities of pure creatine are given by mouth there occurs a slight rise in the excretion of urinary creatinine; much of the creatine, especially if given in large quantity, is excreted as creatine in the urine; the remainder is retained. The retained creatine may be several times larger in quantity than the creatinine normally excreted per day (23, 28).

Moreover, creatinine appears to be quite absent from the blood (1). If we believe that urinary creatinine is derived from the small amount of creatinine that occurs in the muscles, then we would have to face the fact that birds, which show this same muscular creatinine, excrete, not creatinine, but creatine in their urine. The absence of creatinine in detectable quantity in the blood of mammals makes it highly probable that the urinary creatinine is derived from the creatine of the blood, and that in order to excrete this creatine the kidney must first convert it into creatinine. The experiments of Behre and Benedict (1) on this point are extremely suggestive; the ureters of five dogs were ligated, and the effect of this interference of excretion upon the creatine content of the blood estimated. They record a marked increase of blood creatine, and conclude that "creatine accumulates in the blood when the excretory function of the kidney is defective, and we may further conclude that the creatine of the blood is a waste product." We are thus faced with the very curious fact that the kidney is constantly filtering off, as a waste product from the blood, a substance creatine; and that, at the same time, this substance, either on oral or parenteral administration, can be retained by the animal in quantity much greater than that in which it is being excreted. Moreover, while the waste creatine of the blood is excreted as creatinine, that portion of the administered creatine which is excreted is excreted almost entirely as creatine. It is scarcely possible to avoid the conclusion that two forms of creatine exist. Behre and Benedict have already discussed this, and while admitting certain other possibilities, conclude that "it is also possible that the creatine in the circulation differs from ordinary creatine, and is in a combination which can readily be converted into creatinine." The observation of Myers and Fine (24) should also be mentioned, namely, that in muscle tissue autolysing at body temperature, the rate of transformation of creatine into creatinine is over three times as rapid as that occurring in pure solution at the same temperature.

It appears, on this view, that urinary creatinine is to be regarded as a leakage into the blood of the fatigue products that are constantly, even in absolute rest, arising in the muscles. The reason why creatinine excretion does not increase enormously during periods of prolonged work is that under these circumstances the circulation to the muscles automatically adjusts itself to the needs of the muscles, and that under these conditions the oxidative removal of fatigue products proceeds efficiently. Nevertheless, it is generally agreed that the

creatinine excretion is greater in the day than during the resting hours of night. It is also interesting to recall the observation of van Hoogenhuyze and Verploegh (14) that the excretion of creatinine is increased, though not by very much, when living at a high altitude in an oxygen-poor atmosphere; the breathing of pure oxygen at these altitudes again diminishes the creatinine output.

It appears probable, then, that urinary creatinine is simply a leakage product from the muscles, and that it is in a form which the body cannot readily assimilate. The question arises, then, as to the existence of two forms of creatinine, arising through anhydride formation from isocreatine and the basic isomer respectively. Since creatine is such a very feeble base, its capacity for anhydride formation seems doubtful. Johnson (16) indeed has considered sarcous and urinary creatinine to be different; his results have been confirmed by Kemmerich, but denied by others. I have failed to confirm his observation, which constitutes the sharpest difference between the two creatinines, namely, that sarcous creatinine gold chloride crystals are soluble in ether. Taking into consideration the feebly basic properties of creatine, it seems more probable that creatinine formation is always preceded by the movement of the methyl group; further experiment only, however, can decide this point.

## SUMMARY.

1. Frog muscles, fatigued and then immersed in Ringer fluid, liberate considerable quantities of a substance into the surrounding fluid, which, on boiling with acid, yields creatinine. Although the creatine content of fresh muscle is only very slightly less than that of fatigued muscle, yet the quantity of "creatine" liberated by fresh muscle is very much less than that liberated by fatigued muscle. The quantity liberated from fresh muscles is probably proportional to the amount of fatigue they undergo during immersion.

2. Fatigued muscles which have been subjected to a stream of oxygen for five hours liberate very much less "creatine" than fatigued muscles placed for a similar period in a small volume of air.

3. Fatigued muscles liberate "creatine" and lactic acid in equivalent quantities.

4. Muscle creatine probably has a cyclic structure, similar to that of urea. It is inferred that the change which the creatine undergoes during excitation is a splitting open of this ring, resulting in the formation of a free amino group, and that it is this substance which, being formed on the surface of the one excitable membrane, neutralizes the lactic acid produced on the other, and thereby causes muscular relaxation. During oxidative recovery it is reconverted to creatine.

5. The leakage of fatigue products into the blood is discussed. Urinary creatinine appears to be the anhydride of the basic creatine that is constantly leaking into the blood; the rise in the respiratory quotient following upon severe

physical exertion is believed to be due to rapid diffusion into the blood of the lactic acid that is present in large quantity in the muscle throughout the exertion.

In conclusion, I desire to express my deep obligation to Professor Brailsford Robertson for help and advice throughout the work, and for placing at my disposal the facilities of his laboratory. I am also indebted to Professor E. H. Rennie and to Dr. S. W. Pennycuick for advice on some of the chemical points.

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# THE INFLUENCE OF CERTAIN DYES UPON THE MULTIPLICATION OF INFUSORIA (*ENCHELYS*) WITH ESPECIAL REFERENCE TO THE ACRIDINE DYES (ACRIFLAVINE AND PROFLAVINE).

by

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*(Submitted for publication 24th December, 1924.)*

## INTRODUCTION.

When infusoria, or other cells, are isolated from a parent culture or community into freshly-prepared nutrient medium a period of variable duration elapses before multiplication occurs (3, 10, 12, 15). The duration of this period of "lag" varies inversely with the age of the parent culture (12, 14, 15, 16). It is not appreciably affected, in the case of infusoria, at all events, by the number of the individuals which are initially inoculated in the fresh culture-medium. The products which accumulate in thickly-populated culture media and are transferred with the infusoria to the new culture are also without important effect upon the duration of "lag". The effect of various agencies upon the lag-period can, therefore, be ascertained by isolating a group of individuals in a very small drop of the parent-culture fluid (about 3 milligrammes), counting them, and then adding a large drop (about 50 milligrammes) of fresh culture medium, containing one or other of the agencies of which the effect is sought. The duration of lag is then the time which elapses before the number of individuals in the culture undergoes an increase.

Certain dyes, such as scarlet red and basic fuchsin, are reputed to have the power of stimulating the repair of injured mammalian tissues (17). Others, namely, the azine and azonium dyes, such as safranine and janus green, are known to combine with proteolytic enzymes and render them insoluble (8). Since these latter dyes also demonstrably penetrate living cells and stain the mitochondria (4), they might conceivably interfere with the synthesis of proteins which is necessary for the construction of new protoplasm. Certain fluorescent dyes, particularly eosin, are known to destroy lytic substances (11) such as

might conceivably play a role in the multiplication of cells (7). Other dyes, such as methylene blue, are capable of accelerating or retarding oxidations in living tissues (6, 20). It was considered desirable to investigate the influence of representatives of these various groups of dyes upon the lag period and subsequent multiplication of infusoria, since any pronounced effect which might be obtained would probably reveal something of the nature of the chemical processes which underlie and determine these phenomena.

## EXPERIMENTAL.

The organism employed was *Enchelys*, probably *Enchelys farcimen*, or a closely related species, previously utilized by the author in a number of studies on various factors affecting the multiplication of infusoria (14, 15, 16). The organism was originally isolated in 1920 from an infusion of chrysanthemums, and again early in 1924. It is possible that the form isolated in 1924 and employed in this and other investigations conducted during that year (16) is not identical with the form isolated in 1920, for while agreeing in other respects with the description published in 1921 (14) it is smaller in size, typical adult individuals measuring only from 36 $\mu$ to 48 $\mu$ in length, and from 24 $\mu$ to 36 $\mu$ in width, while the organisms isolated in 1920 measured from 50 $\mu$ to 70 $\mu$ in length and from 35 $\mu$ to 50 $\mu$ in width.

The culture medium used in these experiments was prepared by mixing five grammes of chaffed oaten hay with 100 cc. of distilled water, and heating the mixture to boiling in a 200 cc. capacity Erlenmeyer flask. The flask was transferred to a boiling water bath for one hour, and the extract was then filtered, while hot, into a steamed flask. The filtrate was transferred to a 100 cc. graduate and the volume made up to 75 cc., if necessary, by the addition of freshly boiled distilled water. The fluid was then aerated by a stream of air, which passed through a bulb packed with sterilized absorbent cotton, and was introduced at the bottom of the column of liquid through a sterilized glass tube connected with the bulb by a freshly steamed length of rubber tubing. After aeration for one hour, 1 cc. of $\frac{N}{10}$ $NaHCO_3$ solution[1] was added to every 25 cc. of fluid. The cultures were made upon slides provided with a central depression, which lay upon wet pads of filter paper in covered Petri dishes. The slides were sterilized by boiling before use.

The dyes, with the exceptions of scarlet red and phenolphthalein, were dissolved in distilled water in ten times the final concentration desired in the culture, and this solution was heated to boiling. To 10 cc. of culture fluid, prepared in the manner described above, 1 cc. of the dye solution was added. The

[1] The $NaHCO_3$ solution was made up by dissolving the calculated amount of the salt in distilled water, and then saturating this solution with $CO_2$.

dilution of the culture medium which this involved is not sufficient to affect the duration of lag (16).

Scarlet red is not soluble in water. It was found, however, that it is soluble in solutions of saponin or bile salts. Now saponin, even in 0·5 per cent. solution, has no effect upon the duration of lag in subcultures of infusoria (16). The solution of scarlet red employed in these experiments, therefore, was prepared by suspending excess of the solid dye (British drug houses) in a 5 per cent. solution of Merck's saponin and digesting the mixture on a boiling water bath for one hour, with occasional shaking. The mixture was then centrifuged at high speed for fifteen minutes. The supernatant, slightly turbid liquid contained 0·022 per cent. (1 part in 4,500) of scarlet red.[2]

In each experiment with phenolphthalein the highest concentration employed was obtained by adding 1 cc. of a solution of phenolphthalein in 50 per cent. alcohol to 10 cc. of hay infusion. Five per cent. of alcohol by volume is devoid of toxicity for the infusoria employed in these experiments, and has no effect upon the duration of lag (16). The lower concentrations were obtained by diluting the solution of phenolphthalein to one-half, one-fourth, etc., with freshly boiled distilled water and adding 1 cc. of the mixture to 10 cc. of hay infusion.

With exceptions, which will be subsequently noted, subcultures were always inoculated with individuals obtained from parent cultures which were seven days old. The period of lag in subcultures so prepared lies, as a rule, between eighteen and twenty-four hours, but as the variations of lag displayed by individuals isolated from different parent cultures of like age are quite considerable, and depend, moreover, upon temperature, the normal duration of lag was separately ascertained for each of the parent cultures employed.

The following were the results obtained:

With Scarlet Red.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 19 hours. | 24 hours. | 48 hours. |
|---|---|---|---|---|
| Normal Hay Infusion . . . . | 64 | 59 | 70 | — |
| Normal Hay Infusion . . . . | 16 | 15 | 18 | 98 |
| Scarlet Red, 1 in 45,000 . . | 44 | 40 | 38 | 180 |
| Scarlet Red, 1 in 90,000 . . | 23 | 19 | 27 | 138 |
| Scarlet Red, 1 in 180,000 . . | 36 | 36 | 37 | 195 |

The lag period in each culture was, therefore, almost exactly twenty-four hours, and scarlet red, in the concentrations employed, had no appreciable effect, either upon the duration of lag or upon multiplication subsequent to the expiry of lag.

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2 Estimated by diluting 5 cc. to 100 cc. with a mixture of three parts of alcohol and one of chloroform, allowing the precipitate of saponin to settle and comparing the colour of the supernatant liquid in a Bock-Benedict colorimeter with that of a standard solution of scarlet red in alcohol-chloroform mixture containing the same proportion of water (5 per cent. by volume). To obtain a sharp reading in the colorimeter it was necessary to employ yellow light.

WITH PHENOLPHTHALEIN, First Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 21 hours. | 24 hours. | 96 hours. |
|---|---|---|---|---|
| Normal Hay Infusion . . . . | 59 | 52 | 52 | Very numerous |
| Normal Hay Infusion . . . . | 29 | 28 | 30 | Very numerous |
| Phenolphthalein, 1 in 2,000 | 58 | None | None | None |
| Phenolphthalein, 1 in 4,000 | 13 | None | None | None |
| Phenolphthalein, 1 in 8,000 | 46 | None | None | None |
| Phenolphthalein, 1 in 16,000 | 16 | None | None | None |

WITH PHENOLPHTHALEIN, Second Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 21 hours. | 24 hours. | 44 hours. |
|---|---|---|---|---|
| Normal Hay Infusion . . . . | 41 | 59 | 76 | Numerous |
| Normal Hay Infusion . . . . | 36 | 65 | 103 | Numerous |
| Phenolphthalein, 1 in 20,000 | 31 | None | None | None |
| Phenolphthalein, 1 in 40,000 | 47 | None | None | None |
| Phenolphthalein, 1 in 80,000 | 24 | 5 | 2 | 14 |

WITH PHENOLPHTHALEIN, Third Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 19 hours. |
|---|---|---|
| Normal Hay Infusion . . . . | 54 | 99 |
| Normal Hay Infusion . . . . | 62 | 131 |
| Phenolphthalein, 1 in 200,000 | 27 | 39 |
| Phenolphthalein, 1 in 400,000 | 61 | 130 |
| Phenolphthalein, 1 in 800,000 | 27 | 58 |

It is evident that phenolphthalein in concentrations above 1 in 80,000 is lethal. At this concentration prolongation of lag was observed, but the decrease of the number inhabiting this culture between the twenty-first and twenty-fourth hours, while the control cultures were increasing in population, suggests that lag was prolonged through the killing of susceptible individuals, while those which were less susceptible survived and multiplied. Concentrations of 1 in 200,000 and less exerted no appreciable effect, either upon lag or upon subsequent multiplication. It was observed that the cultures containing phenolphthalein turned pink as multiplication proceeded, indicating an increase of $P_H$ to at least 8·3 (2).

WITH METHYLENE BLUE (Gruebler's rect. nach Ehrlach). First Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 20 hours. | 25 hours. | 48 hours. | 72 hours. |
|---|---|---|---|---|---|
| Normal Hay Infusion . . . . | 8 | 20 | 45 | 479 | — |
| Methylene Blue, 1 in 10,000 | 9 | 8 | 12 | 63 | 76 |
| Methylene Blue, 1 in 20,000 | 8 | 16 | 36 | 380 | — |
| Methylene Blue, 1 in 40,000 | 21 | 55 | — | — | — |

WITH METHYLENE BLUE, Second Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 19 hours. | 23 hours. | 70 hours. |
|---|---|---|---|---|
| Normal Hay Infusion . . . . | 31 | 19 | 22 | Numerous |
| Normal Hay Infusion . . . . | 33 | 27 | 34 | Numerous |
| Methylene Blue, 1 in 200,000 | 36 | 32 | 34 | Numerous |
| Methylene Blue, 1 in 800,000 | 23 | 23 | 21 | Numerous |
| Methylene Blue, 1 in 1,600,000 | 29 | 21 | 21 | Numerous |

There is evident prolongation of lag and slowing of subsequent multiplication in the culture containing 1 in 10,000 of methylene blue. Concentrations which exceed this are toxic, while half this concentration exerts no appreciable effect at all upon lag or upon subsequent multiplication. The presence of this hydrogen-acceptor, and the presumable acceleration of certain oxidations which it entails (6, 20), therefore, does not at any concentration below 1 in 10,000 abbreviate the phenomena which underlie and determine the duration of lag and of the subsequent inter-divisional periods, notwithstanding the fact that the synthesis of nuclear material is dependent upon oxidations (7).

It was noted that the toxicity of methylene blue for *Enchelys* is much higher in distilled water buffered with $NaHCO_3$ than it is in hay infusion which is similarly buffered. In distilled water containing 4 cc. per cent. of tenth-normal $NaHCO_3$, methylene blue kills these infusoria in from five to ten minutes at a dilution of 1 to 100,000. They survive indefinitely in solutions containing 1 in 200,000 or less.

WITH JANUS GREEN (Janus grün B, Lucius and Brüning). First Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 18 hours. | 66 hours. | 90 hours. | 120 hours. | 144 hours. | 168 hours. |
|---|---|---|---|---|---|---|---|
| Normal Hay Infusion . . | 6 | 14 | Numerous | Numerous | — | — | — |
| Janus Green, 1 in 10,000 | 9 | 1 | 3 | 10 | 17 | 17 | 20 |
| Janus Green, 1 in 20,000 | 7 | 5 | 67 | Numerous | — | — | — |
| Janus Green, 1 in 40,000 | 5 | 3 | 37 | Numerous | — | — | — |

WITH JANUS GREEN, Second Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 22 hours. | 27 hours. |
|---|---|---|---|
| Normal Hay Infusion. . . | 43 | 74 | 146 |
| Normal Hay Infusion . . . | 21 | 37 | 77 |
| Janus Green, 1 in 100,000 | 22 | 21 | 27 |
| Janus Green, 1 in 100,000 | 38 | 32 | 52 |
| Janus Green, 1 in 200,000 | 20 | 25 | 50 |
| Janus Green, 1 in 400,000 | 24 | 31 | 56 |
| Janus Green, 1 in 400,000 | 42 | 78 | 143 |
| Janus Green, 1 in 800,000 | 15 | 22 | 40 |
| Janus Green, 1 in 1,600,000 | 27 | 51 | 82 |

Concentrations of janus green in excess of 10,000 are lethal. It will be noted that at this concentration an exceptionally high mortality occurs among the infusoria introduced into the culture, and the subsequent development of those which survive is enormously retarded, the population after increasing very slowly from the first to the fifth day, remaining almost constant until the seventh day. Less pronounced toxicity and retardation of multiplication was evidenced in more dilute solutions. In the higher concentrations (1 in 40,000 and upwards) the effect on the duration of lag is rendered uncertain by the excessive mortality among the individuals isolated into the cultures. Thus the number inhabiting the culture which contained 1 in 20,000 janus green, although less at eighteen hours than it was initially, may have been still less at some time prior to eighteen hours, owing to the toxicity of the culture fluid for certain sensitive individuals, so that the duration of lag, for all we can tell, may actually, as in the controls in this instance, have been less than eighteen hours. Slight prolongation of lag, however, as well as definite slowing of development, occurred in the cultures containing 1 in 100,000 and 1 in 200,000 of the dye. Dilutions greater than this were without effect.

As in the case of methylene blue, it was found that janus green is definitely more toxic in buffered distilled water than in hay infusion similarly buffered by the addition of $NaHCO_3$. Thus a concentration of 1 in 20,000 in buffered distilled water was lethal in two hours, while 1 in 40,000 killed nearly all of the infusoria introduced into it within the same period. In 1 in 80,000 solution about half the infusoria introduced into the mixture were dead at the end of two hours.

WITH SAFRANINE (Gruebler), First Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 24 hours. | 43 hours. |
|---|---|---|---|
| Normal Hay Infusion . . . | 11 | 12 | 66 |
| Safranine, 1 in 10,000 . . . | 7 | 3 (dividing) | 24 |
| Safranine, 1 in 20,000 . . . | 13 | 9 | 41 |
| Safranine, 1 in 40,000 . . . | 9 | 8 (dividing) | 33 |

WITH SAFRANINE, Second Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 20 hours. | 68 hours. |
|---|---|---|---|
| Normal Hay Infusion . . . | 16 | 26 | Numerous |
| Normal Hay Infusion . . . | 5 | 10 | Numerous |
| Safranine, 1 in 25,000 . . . | 7 | 7 | 280 |
| Safranine, 1 in 50,000 . . . | 6 | 11 | Numerous |
| Safranine, 1 in 100,000 . . . | 7 | 7 | Numerous |

WITH SAFRANINE, Third Experiment.

| | Original No. in Culture. | Number inhabiting Culture after 22 hours. | 24 hours. | 72 hours. |
|---|---|---|---|---|
| Normal Hay Infusion . . . | 19 | 19 | 19 (dividing) | Numerous |
| Normal Hay Infusion . . . | 23 | 26 | 30 | Numerous |
| Safranine, 1 in 100,000 . . | 17 | 17 | 17 | Much less numerous |
| Safranine, 1 in 100,000 . . | 12 | 13 (dividing) | 15 | Moderately numerous |
| Safranine, 1 in 200,000 . . | 28 | 30 | 38 | Numerous |
| Safranine, 1 in 200,000 . . | 19 | 23 | 29 | Numerous |
| Safranine, 1 in 400,000 . . | 30 | 29 (dividing) | 43 | Numerous |
| Safranine, 1 in 800,000 . . | 36 | 42 | 59 | Numerous |
| Safranine, 1 in 1,600,000 . . | 23 | 28 | 36 | Numerous |
| Safranine, 1 in 3,200,000. . | 29 | 33 | 47 | Numerous |

It is evident that some prolongation of lag is induced by concentrations of safranine exceeding 1 in 100,000, and subsequent multiplication is delayed by this and higher concentrations. In no concentration employed is lag prolonged or multiplication retarded to anything like the extent that they are in solutions containing similar concentrations of janus green. Hay infusions containing 1 in 10,000 or more of safranine deposit excess of undissolved dye on standing, and, therefore, under the conditions which obtain in these cultures, presumably represent saturated solutions.

The toxicity of safranine in buffered distilled water is far greater than it is is similarly buffered hay infusion. Thus 1 in 64,000 in distilled water kills the majority of infusoria introduced into it within one and a half hours. Higher concentrations are immediately lethal.

WITH ACRIFLAVINE (Methyldiamino Acridinium Hydrochloride, Boots). First Experiment.

| | Original No. in Culture. | Number inhabiting Culture after 19 hours. | 24 hours. | 48 hours. |
|---|---|---|---|---|
| Normal Hay Infusion . . . | 64 | 59 | 70 | 180 |
| Normal Hay Infusion . . . | 16 | 15 | 18 | 98 |
| Acriflavine, 1 in 1,000 . . . | 33 | None | None | None |
| Acriflavine, 1 in 2,000 . . . | 25 | None | None | None |
| Acriflavine, 1 in 4,000 . . . | 32 | None | None | None |
| Acriflavine, 1 in 8,000 . . . | 36 | 9 | 4 | 1 |

This experiment shows that concentrations of acriflavine in excess of 1 in 8,000 are rapidly lethal, and 1 in 8,000 itself is lethal, but its action is curiously

prolonged, individuals gradually dying throughout forty-eight hours after their introduction into the fluid. The toxicity of acriflavine in buffered distilled water is far greater than in similarly buffered hay infusion, as the following experiment shows:

WITH ACRIFLAVINE, Second Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 21 hours. | 45 hours. |
|---|---|---|---|
| $\frac{N}{250}$ Sodium bicarbonate . . . . . | 25 | 19 | 19 |
| Same containing 1 in 50,000 Acriflavine | 24 | None | None |
| Same containing 1 in 100,000 Acriflavine | 21 | None | None |
| Same containing 1 in 200,000 Acriflavine | 21 | None | None |

The toxicity of acriflavine in distilled water varies, however, with the age of the parent culture from which the organisms are derived, those derived from younger cultures being more resistant. It is also diminished by the addition of buffer salts (sodium carbonate or bicarbonate). The following experiment illustrates these facts:

WITH ACRIFLAVINE, Third Experiment. Parent Culture three days old.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 19 hours. | 67 hours. |
|---|---|---|---|
| Distilled water . . . . . | 22 | 22 | 41 |
| Same containing 1 in 50,000 Acriflavine | 10 | None | None |
| Same containing 1 in 100,000 Acriflavine | 13 | None | None |
| $\frac{N}{250}$ $NaHCO_3$ containing 1 in 50,000 Acriflavine | 15 | 2 | None |
| Same containing 1 in 100,000 Acriflavine | 16 | 7 | 9 |
| $\frac{N}{250}$ $Na_2CO_3$ containing 1 in 50,000 Acriflavine | 12 | None | None |
| Same containing 1 in 100,000 Acriflavine | 14 | 7 | 9 |

The higher toxicity of acriflavine in distilled water containing no buffer salts may perhaps be accounted for by the acid reaction communicated to the water by the dye. In the buffered mixtures this could not have contributed to their toxicity, because a 1 in 1,000 solution of acriflavine contains in all less than $\frac{N}{250}$ of combined hydrochloric acid, and the solutions which are lethal must, therefore, have contained a large proportion of the unneutralized buffer.

WITH ACRIFLAVINE, Fourth Experiment.

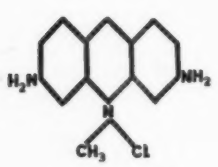
| Culture Fluid. | Original No. in Culture. | Number inhabiting 21 hours. | 24 hours. | Culture after 4 days. |
|---|---|---|---|---|
| Normal Hay Infusion . . . . . | 59 | 52 | 52 | Very numerous |
| Normal Hay Infusion . . . . . | 29 | 28 | 30 | Very numerous |
| Acriflavine, 1 in 10,000 . . . | 56 | 26 | 20 | 4 |
| Acriflavine, 1 in 20,000 . . . | 25 | 20 | 18 | 15 |
| Acriflavine, 1 in 40,000 . . . | 28 | 25 | 28 | 29 |

WITH ACRIFLAVINE, Fifth Experiment.

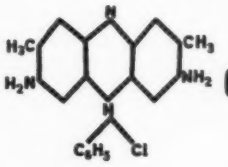
| Culture Fluid. | Original No. in Culture. | Number inhabiting 21 hours. | 24 hours. | Culture after 2 days. | 7 days. |
|---|---|---|---|---|---|
| Normal Hay Infusion . . | 41 | 59 | 76 | About 800 | — |
| Normal Hay Infusion . . | 36 | 65 | 103 | Numerous | — |
| Normal Hay Infusion . . | 30 | 56 | 72 | Numerous | — |
| Acriflavine, 1 in 100,000 | 27 | 26 | 26 | 26 | 25 |
| Acriflavine, 1 in 200,000 | 19 | 18 | 19 | 19 | — |
| Acriflavine, 1 in 400,000 | 14 | 14 | 14 | 43 | Numerous |

These experiments illustrate an extraordinary feature in the effect of acriflavine upon these organisms. While concentrations ranging from 1 in 8,000 to 1 in 20,000 in buffered hay infusion are very gradually lethal, individuals dying at long intervals without reproduction of others to replace them, dilutions ranging from 1 in 20,000 to 1 in 200,000, while non-toxic in other respects, nevertheless completely inhibit reproduction, the population remaining absolutely stationary, for example, in 1 in 100,000 acriflavine, for no less than a week. No other dye among those investigated, with the exception of the closely related proflavine (diamino acridinium sulphate), exerts any effect at all comparable with this. Janus green has a somewhat similar effect, as we have seen, in 1 in 10,000 dilution, but the range of concentrations over which this effect is exerted in the case of janus green is very narrow, for 1 in 8,000 solutions are lethal and 1 in 20,000 solutions do not inhibit reproduction. The fact that janus green, even within this comparatively narrow zone of concentrations, can also inhibit reproduction over long periods without other evident injury to the organisms, however, is of interest because there is this extent of structural resemblance between janus green and acriflavine, that both contain nitrogen attached to two benzene rings, although janus green contains two such nitrogens, and acriflavine contains only one nitrogen atom of this type. On the other hand, safranine, which is closely related to janus green, exhibited no such effect at any dilution which it was found practicable to employ.

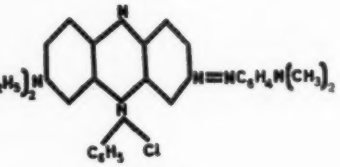
The fact that acriflavine restrains or prevents reproduction over such an extraordinarily wide range of otherwise non-toxic concentrations forcibly suggests a specific interference with some process which underlies and determines the multiplication of cells. It will be observed (fifth experiment, above) that while 1 in 400,000 acriflavine permits reproduction at some time after twenty-four hours, the duration of lag is prolonged beyond that period, whereas, in this




Acriflavine




Safranine




Janus green B

instance, in the absence of acriflavine, the lag period was less than twenty-one hours. In some instances a dilution of 1 in 400,000 did not appreciably prolong the lag period, but, nevertheless, subsequent multiplication was greatly retarded by this and even higher dilutions, as the following experiment shows:

WITH ACRIFLAVINE, Sixth Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 19 hours. | 24 hours. | 44 hours. | 68 hours. | Divisions which occurred between 19 and 44 hours. |
|---|---|---|---|---|---|---|
| Normal Hay Infusion . . | 44 | 37 | 51 | 238 | Very numerous | 2·6 |
| Normal Hay Infusion · · | 24 | 22 | 23 | 114 | Very numerous | 2·3 |
| Acraflavine, 1 in 200,000 | 12 | 9 | 9 | 13 | 30 | 0·4 |
| Acriflavine, 1 in 200,000 | 34 | 26 | 33 | 47 | 126 | 0·8 |
| Acriflavine, 1 in 400,000 | 15 | 10 | 14 | 26 | — | 1·6 |
| Acriflavine, 1 in 500,000 | 42 | 35 | 38 | 95 | — | 1·7 |
| Acriflavine, 1 in 600,000 | 40 | 34 | 36 | 94 | — | 1·8 |
| Acriflavine, 1 in 600,000 | 53 | 53 | 54 | 147 | — | 1·8 |
| Acriflavine, 1 in 700,000 | 20 | 19 | 23 | 54 | — | 1·8 |
| Acriflavine, 1 in 800,000 | 12 | 11 | 14 | 43 | — | 2·0 |

In all these cultures the duration of lag lay between nineteen and twenty-four hours, or was almost precisely twenty-four hours, because in those cultures in which increase of numbers had not actually occurred divisions could be seen to be proceeding. Nevertheless, although acriflavine at these dilutions does not

affect the duration of lag, the duration of inter-divisional periods is manifestly prolonged and nearly in direct proportion to the concentration of the acriflavine. Even higher dilutions (one in a million) are capable of delaying multiplication, as the following experiment shows:

WITH ACRIFLAVINE, Seventh Experiment.

| Culture Fluid. | Original No. in Culture. | Number inhabiting Culture after 19 hours. |
|---|---|---|
| Normal Hay Infusion . . . . | 54 | 99 |
| Normal Hay Infusion . . . . | 62 | 131 |
| Acriflavine, 1 in 1,000,000 . . | 27 | 39 |
| Acriflavine, 1 in 2,000,000 . . | 61 | 130 |
| Acriflavine, 1 in 4,000,000 . . | 27 | 58 |

Thus, while the individuals introduced into normal hay infusion, or into hay infusion containing 1 in 2,000,000 or less of acriflavine, doubled their numbers in nineteen hours, in hay infusion containing 1 in 1,000,000 of acriflavine the numbers had only increased 50 per cent. At no dilution from 1 in 10,000 to 1 in 4,000,000 was any abbreviation of lag or acceleration of division rates observed. The effect, when the dye is sufficiently concentrated to exert any effect at all, is wholly one of retardation.

We have seen that concentrations of acriflavine in excess of 1 in 400,000 cause prolongation of lag, which becomes virtually of indefinite duration at concentrations in excess of 1 in 100,000. Now it has been shown elsewhere (16) that a previous period of acclimatization of infusoria (*Enchelys*) to the new culture fluid (by mixture of the parent culture with an equal volume or twice its volume of fresh culture fluid one hour before withdrawal of the individuals destined to populate the subculture) results in abbreviation of the period of lag, reckoned from the moment at which acclimatization began. The following experiment was designed to ascertain whether this process of acclimatization has any affect upon the prolongation of lag which is induced by the presence of acriflavine in the culture fluid.

To a parent culture, seven days old, was added twice its original volume of fresh hay infusion. Sample groups of organisms were immediately withdrawn ("not acclimatized") and introduced into normal hay infusion, 1 in 100,000 and 1 in 200,000 acriflavine respectively. Fresh samples were withdrawn after one, three, and five hours respectively, and introduced into the same culture media. Time was estimated from the moment of mixing the parent culture with fresh culture fluid.

WITH ACRIFLAVINE, Eighth Experiment.

| Culture fluid. | Acclimatization. | Original No. in Culture. | Number inhabiting culture after 23 hours. | 26 hours. | 3 days. | 4 days. | 5 days. | 6 days. | 7 days. | 8 days. |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal hay infusion | None | 9 | 26 | 38 | Very numerous | — | — | — | — | — |
| Normal hay infusion | 1 hour | 18 | 72 | 119 | Very numerous | — | — | — | — | — |
| Normal hay infusion | 3 hours | 13 | 44 | 72 | Very numerous | — | — | — | — | — |
| Normal hay infusion | 5 hours | 12 | 35 | 53 | Very numerous | — | — | — | — | — |
| Acriflavine: | | | | | | | | | | |
| 1 in 100,000 | None | 15 | 14 | 14 | 14 | 11 | 12 | 10 (One dividing) | 11 (One dividing) | 14 (Three dividing) |
| 1 in 100,000 | 1 hour | 11 | 11 | 11 | 15 | 17 | 16 | 26 | 48 | Not counted |
| 1 in 100,000 | 3 hours | 11 | 11 | 11 | 13 | 24 | 24 | 34 | 62 | Not counted |
| 1 in 100,000 | 5 hours | 12 | 11 | 11 | 11 (One dividing) | 15 | 16 | 17 | 34 | Not counted |
| 1 in 200,000 | None | 8 | 8 | 8 | 178 | — | — | — | — | — |
| 1 in 200,000 | 1 hour | 32 | 40 | 52 | Moderately numerous | — | — | — | — | — |
| 1 in 200,000 | 3 hours | 7 | 7 (One dividing) | 12 | 141 | — | — | — | — | — |
| 1 in 200,000 | 5 hours | 11 | 20 | 23 | 282 | — | — | — | — | — |
| Acriflavine, 1 in 100,000, which had stood at room temperature for 24 hours . . . . . | None | 11 | 10 | 10 | 10 | — | — | — | — | — |
| Acriflavine, 1 in 100,000, which had stood at room temperature for 24 hours . . . . . | 1 hour | 25 | 25 | 25 | 37 | — | — | — | — | — |
| Acriflavine, 1 in 100,000, which had stood at room temperature for 24 hours . . . . . | 3 hours | 18 | 17 | 17 | 19 | — | — | — | — | — |
| Acriflavine, 1 in 100,000, which had stood at room temperature for 24 hours . . . . . | 5 hours | 12 | 12 | 12 | 20 | — | — | — | — | — |

In normal hay infusion, it will be observed, the duration of lag was less than twenty-three hours, for by that time unacclimatized individuals had multiplied to three times their number: those which had been acclimatized for one hour had multiplied to four times their number, those which had been acclimatized three and five hours to 3·4 and 3·0 their number respectively. In 1 in 100,000 acriflavine, on the contrary, the lag period exceeded three days for unacclimatized individuals, and divisions were just beginning at the end of the third day in individuals which had been acclimatized. In 1 in 200,000 acriflavine lag exceeded twenty-six hours in unacclimatized individuals, but in acclimatized individuals the duraton of lag was almost exactly twenty-three hours, subsequent development being very decidedly retarded. The effect of acclimatization is to abbreviate lag in acraflavine cultures, as in normal cultures, therefore, but the extent of abbreviation attainable by this means is evidently not great. It will be observed that there is an optimal period of acclimatization, in this case, between one and three hours, after which further acclimatization, so far from causing additional abbreviation of lag, lengthens it again.

It appeared possible at first that the excessive duration of lag in cultures containing acriflavine might be due to nothing more than suppression of growth of bacteria, due to its reputed antiseptic action, for, whether infusoria actually digest bacteria, or, as Peters has suggested (13), subsist upon elementary foodstuffs, such as ammonium salts and the simpler compounds of carbon, in either case the presence of bacteria must lead to an increase of available foodstuffs, in the first by providing in themselves a source of nutrition, and in the second by breaking down complex nitrogenous and carbohydrate compounds, and rendering them available for consumption by the infusoria. Thus suppression of bacterial growth, if this were actually achieved by the concentrations of acriflavine which were employed, might very intelligibly lead to suppression of the multiplication of the infusoria as well. This conjecture was speedily rendered untenable, however, by observation of cultures containing 1 in 100,000 of acriflavine, in which reproduction of infusoria was totally inhibited over several days, during which the cultures were manifestly swarming with bacteria.

In the experiment just described, a mixture of fresh hay infusion with 1 in 100,000 acriflavine was allowed to stand at room temperature (about 16°C.) for twenty-four hours. By that time it had accumulated a considerable population of bacteria, but, as the experiment shows, the additional nutrients thus presumably rendered available for consumption by the infusoria did not in any instance diminish the duration of lag in comparison with that observed in 1 in 100,000 solutions of acriflavine in fresh hay infusion.

It is evident that in concentrations which suffice to check the reproduction of infusoria reproduction of bacteria in hay infusion is not inhibited. According to Browning, Gulbransen, Kennaway, and Thornton (1), the lethal concentration

of acriflavine in water containing 0·7 per cent. peptone is 1 in 1,300 for *Bacillus coli communis*, and 1 in 20,000 for *Staphylococcus aureus*. These concentrations lie far above those found effective in checking the multiplication of infusoria, but it is still possible that the slowly *lethal* effect of 1 in 10,000 and higher concentrations of acriflavine in hay infusion may partly be accounted for by starvation of the organisms due to a deficiency of bacterial population in the cultures.

Even when division does occur in 1 in 100,000 acriflavine, in consequence of previous acclimatization, it only occurs in a small proportion of the organisms at any one time, so that the period required to complete the division of all the individuals in the culture is very long and the interdivisional period which follows is longer still. If, however, individuals be isolated from such a culture, in which divisions are occurring, into fresh hay infusion not containing any acriflavine, then division of these individuals and their offspring proceeds at a fairly rapid and uniform rate. The following experiment illustrates this fact:

With Acriflavine, Ninth Experiment.

| Age of the parent Culture. | Population of parent Culture in 1 in 100,000 Acriflavine. | Population of Subculture in normal hay infusion inoculated on third day with a single individual from the parent Culture. |
|---|---|---|
| Initial population . . . | 11 | — |
| 1 day . . . . . | 11 | — |
| 3 days . . . . . | 13 | 1 (subculture started) |
| 3 days + 20 hours | — | 2 |
| 4 days . . . . . | 24 | 4 |
| 5 days . . . . . | 24 | 36 |
| 6 days . . . . . | 34 | 225 |
| 7 days . . . . . | 62 | Very numerous |

Thus during the twenty-four hours which elapsed after inoculation of the subculture the individuals inhabiting the parent culture divided once, while that inhabiting the subculture had divided twice. During the succeeding twenty-four hours three divisions had occurred in the subculture and none at all in the parent culture. Twenty-four hours later 2·5 divisions had occurred in the subculture and one division was not quite completed in the parent culture. This experiment shows quite clearly that acriflavine in this dilution is not toxic for the infusoria in the sense of inflicting any permanent injury upon them. In fact, there is no evidence at present of any injury other than retardation of multiplication. The capacity for multiplication is retained, however, and finds expression as soon as the inhibiting influence of acriflavine is removed. This experiment shows, moreover, that the effect of acriflavine is not to prevent cell-division, which is still possible, but to retard those preliminary processes which occur in the interdivisional period which, in consequence, is greatly increased in duration. But

since the amount of this increase differs in different individuals, the divisions, when they occur, are scattered over a longer period than usual before the fission of all of the individuals which inhabit the culture has become accomplished. We may also infer that the occurrence of divisions after a very considerable lapse of time in cultures containing 1 in 100,000 acriflavine is not due to decomposition of the acriflavine and consequent insufficiency of the remainder to inhibit multiplication, for, in the first place, in the unacclimatized cultures, in which there is no reason to suppose that decomposition of the dye would be less rapid, multiplication is inhibited long after its occurrence in acclimatized cultures. The persistence of the effects of acclimatization is, indeed, most remarkable, when we consider that multiplication may be influenced six days later by preliminary acclimatization of only three or five hours' duration. In the second place, in those acclimatized cultures in which division does occur, the subsequent interdivisional period is so greatly prolonged as to show that the inhibiting effect of acriflavine is still being exerted.

The data suggest that individuals arising in acriflavine cultures may possibly be more susceptible to the toxic action of the dye than the parent forms or, what is more probable, that the process of division in the presence of acriflavine itself leads to death of the organisms.[3] For in the culture in 1 in 100,000 acriflavine, which was not acclimatized, the numbers decrease slightly between the third and fourth days, when divisions are beginning to occur in the acclimatized cultures, and again between the fifth and the sixth days at which time division could be seen to be occurring in this culture.

In the preceding experiment the individual employed to inoculate the subculture was removed from the parent culture just at the moment when, notwithstanding the presence of acriflavine, the organisms inhabiting the culture were just about to divide. If, however, organisms removed from a parent culture in normal hay infusion are exposed for varying periods to hay infusion containing acriflavine, and then transferred to normal hay infusion, the duration of lag and of subsequent interdivisional periods is definitely prolonged in proportion to the duration of the preliminary exposure to acriflavine. The following experiment illustrates this fact.

A pipette of the type employed in counting white blood corpuscles was filled to the 0·5 mark with fresh hay infusion containing 1 in 50,000 of acriflavine. A seven-day-old culture of *Enchelys* in normal hay infusion was then sucked up into the pipette until the combined contents reached the 1·0 mark. The total contents were then discharged into the central depression on a slide, and mixed by blowing two or three bubbles of air through them. From time to time small samples (0·003 cc.) were removed and inoculated into 0·05 cc. of normal hay infusion. The following were the results obtained:

[3] For it will be shown (Eleventh Experiment) that individuals which have been recently produced in normal hay infusion are not *more* but *less* sensitive to the action of Acriflavine.

WITH ACRIFLAVINE, Tenth Experiment.

| Time of preliminary exposure to 1 in 100,000 Acriflavine. | Initial Number. | Number inhabiting Subculture (time computed from moment of mixture of parent Culture with 1 in 50,000 Acriflavine) At 24 hours. | At 28 hours. | At 47 hours. | At 53 hours. | At 71 hours. |
|---|---|---|---|---|---|---|
| 5 minutes . . . | 23 | 18 | 21 | 109 | Not counted | Numerous |
| 100 minutes . . . | 15 | 10 | 11 | 21 | 37 | 228 |
| 170 minutes . . . | 15 | 13 | 14 | 21 | 42 | 194 |
| 260 minutes . . . | 17 | 12 | 12 | 19 | 25 | 119 |

The contrast between the results obtained in this and the preceding experiment is most remarkable, for whereas three days' exposure to acriflavine did not retard subsequent development in normal hay infusion, so brief an exposure as 100 minutes reduced the multiplicative rate of individuals subsequently returned to normal hay infusion to one-sixth of the rate which obtained after an exposure of only five minutes (computed in terms of divisions occurring in forty-seven hours). We cannot suppose that infusoria which have been exposed to acriflavine for long periods of time become thereby habituated to it, for we have seen that when, after some days, division does occur in acriflavine cultures the subsequent interdivisional period is again enormously prolonged, showing that the dye still exerts its inhibitive effect upon the interdivisional processes. It appears evident, therefore, that at the moment of division a fresh start is made, so that if the organism is now removed from the influence of acriflavine the previous exposure goes for little or nothing, whereas if the organism is exposed to acriflavine *during an interdivisional period* a prolonged retardation of interdivisional processes occurs which is proportional to the duration of exposure. We see, once more, that the action of acriflavine is to retard interdivisional processes, and not the process of division itself. Moreover, at the moment of division the previous effects of acriflavine are nullified.

In preceding experiments, with the exception of the subculture in the ninth experiment, the organisms inoculated into the various culture media were invariably obtained from seven-day-old parent cultures, in which division had ceased. The following experiment was designed to ascertain the influence of the age of the parent culture upon the capacity of acriflavine to inhibit reproduction in subcultures inoculated from them. To parent cultures which were one, two, three, four, and seven days old, respectively, were added equal volumes of fresh hay infusion and, after one hour's acclimatization, about 0·003 cc. was removed from each and inoculated into 0·05 cc. of normal hay infusion or of hay infusion containing 1 in 50,000 or 1 in 100,000 acriflavine. The following were the results obtained, time being reckoned from the beginning of the period of acclimatization. Since the numbers of individuals contained in the original inoculum differed so

greatly both the populations of the cultures and the divisions of the original individuals and their offspring to which these populations correspond, are given in the table. When the population underwent decrease during the first twenty-four hours the division rate is computed on the basis of the minimal observed population. All results, therefore, are minimal, since some organisms may have died and have been replaced by others prior to the minimal count (or after, in case the minimal count was the initial one).

WITH ACRIFLAVINE, Eleventh Experiment.

| Culture Fluid. | Age of Parent Culture. | Initial Number. | Population of Subculture after | | | Divisions which occurred during | | |
|---|---|---|---|---|---|---|---|---|
| | | | 24 hours. | 27 hours. | 75 hours. | 24 hours. | 27 hours. | 75 hours. |
| Normal hay infusion | 7 days | 9 | 81 | 125 | Numerous | 3·1 | 3·7 | — |
| Normal hay infusion | 4 days | 7 | 73 | 114 | Numerous | 3·3 | 4·0 | — |
| Normal hay infusion | 3 days | 13 | 143 | 252 | Numerous | 3·4 | 4·2 | — |
| Normal hay infusion | 2 days | 4 | 137 | 255 | Numerous | 5·1 | 6·0 | — |
| Normal hay infusion | 1 day | 1 | 42 | 55 | 371 | 5·3 | 5·4 | 8·5 |
| Acriflavine: | | | | | | | | |
| 1 in 100,000 | 7 days | 12 | 12 | 17 | 28 | 0·0 | 0·4 | 1·3 |
| 1 in 100,000 | 4 days | 9 | 12 | 15 | 26 | 0·3 | 0·7 | 2·9 |
| 1 in 100,000 | 3 days | 5 | 3 | 4 | 12 | 0·0 | 0·3 | 2·0 |
| 1 in 100,000 | 2 days | 7 | 21 | 22 | 24 | 1·5 | 1·6 | 1·7 |
| 1 in 100,000 | 1 day | 2 | 8 | 8 | 7 | 2·0 | 2·0 | 1·8 |
| 1 in 50,000 | 7 days | 18 | 12 | 15 | 20 | 0·0 | 0·3 | 0·7 |
| 1 in 50,000 | 4 days | 6 | 4 | 4 | 7 | 0·0 | 0·0 | 1·8 |
| 1 in 50,000 | 3 days | 10 | 8 | 9 | 13 | 0·0 | 0·1 | 0·6 |
| 1 in 50,000 | 2 days | 4 | 4 | 6 | 9 | 0·0 | 0·3 | 1·1 |
| 1 in 50,000 | 1 day | 2 | 6 | 6 | 7 | 1·5 | 1·5 | 1·8 |

It is evident that, with decreasing age of the parent culture, just as multiplicative rate increases in normal hay infusion, so, but to a less pronounced degree, does it increase in culture fluids which contain acriflavine. The extent to which this occurs also varies with the concentration of acriflavine. Thus the increase of division-rate with diminishing age of the parent culture is evident and fairly regular in 1 in 100,000 acriflavine, the maximum rate being attained in the subculture from the one-day-old parent culture. In 1 in 50,000 acriflavine the increase in division-rate, with diminishing age of the parent culture, is very slight and irregular until the age of the parent culture falls from two days to one day, when a very considerable increase of division-rate occurs.

The closely related acridine derivative, proflavine (diamino acridinium sulphate) has a similar effect in inhibiting the reproduction of infusoria (*Enchelys*), but the effect is exerted over a narrower range of concentrations. In distilled water buffered with $NaHCO_3$ it is eight times as toxic for these

organisms as acriflavine, concentrations of 1 in 80,000 being lethal within ninety minutes. This fact appeared to be very remarkable, because the toxicity of proflavine for higher animals is stated to be much less than that of acriflavine. It was deemed advisable to confirm this statement. A series of young mice, weighing between 20 and 25 grammes, received the following doses of acriflavine hypodermically administered in aqueous solution: 10 mg., 5 mg., 2·5 mg., 1·875 mg., 1·25 mg., and 0·625 mg. All the mice, except that receiving the lowest dosage, died within twenty-four hours, the most striking premortal symptom being excessive slowness of the respiratory movements, indicating paralysis of the respiratory centre (19). Subsequent experiments indicated that the lethal dose for mice of this weight is almost exactly 1 milligramme, but smaller doses, repeated daily, may be lethal after two or three days, indicating that the dye is not completely excreted in twenty-four hours (19). A similar series of mice received the following doses of proflavine: 5 mg., 2·5 mg., 1·25 mg., 1·0 mg., 0·5 mg., and 0·25 mg. Of these, only the mouse which received the highest dosage died. Evidently, therefore, acriflavine is between two and a half and five times as toxic for mice as proflavine, while, in buffered distilled water, proflavine is eight times as toxic for infusoria as acriflavine. The lethal concentration of proflavine in hay infusion has not been accurately determined, but it is certainly much lower than the lethal concentration of acriflavine. Thus, in one experiment, concentrations of 1 in 100,000 and over in hay infusion were found to be lethal after twenty-four hours. In another experiment (see below) a concentration of 1 in 100,000 was not lethal, but inhibited reproduction for forty-eight hours.

WITH PROFLAVINE (Diamino Acridinium Sulphate, Boots).

| Culture Fluid. | Initial No. in Culture. | Number inhabiting Culture after 25 hours. | 28 hours. | 46 hours. |
|---|---|---|---|---|
| Normal hay infusion . . . | 21 | 20 | 24 | 120 |
| Normal hay infusion . . . | 10 | 6 | 9 | 58 |
| Proflavine, 1 in 100,000 | 11 | 5 | 5 | 5 |
| Proflavine, 1 in 100,000 | 10 | 6 | 6 | 8 |
| Proflavine, 1 in 200,000 | 11 | 8 | 8 | 25 |
| Proflavine, 1 in 200,000 | 11 | 6 | 6 | 22 |
| Proflavine, 1 in 400,000 | 31 | 21 | 24 | 120 |
| Proflavine, 1 in 800,000 | 9 | 6 | 6 | 36 |
| Proflavine, 1 in 1,600,000 | 15 | 12 | 11 | 38 |
| Proflavine, 1 in 3,200,000 | 18 | 18 | 22 | 128 |

Thus the action of proflavine is similar to that of acriflavine, but concentrations below 1 in 200,000 do not delay multiplication, while concentrations lying not far above 1 in 100,000 are toxic. The effect is, therefore, exerted over a much narrower range of dilutions than that of acriflavine.

## SUMMARY OF THE EXPERIMENTAL RESULTS.

Scarlet red in the concentrations employed (1 in 45,000 and less) exerted no effect upon the duration of lag in cultures of *Enchelys,* or upon subsequent multiplication. The lethal concentration was not determined.

Phenolphthalein induces no prolongation of lag or delay of subsequent multiplication in any non-lethal concentration.

Methylene blue and safranine cause slight prolongation of lag in concentrations just below those which are lethal. Lower concentrations have no effect.

Janus green, in 1 in 10,000 concentration, causes very great prolongation of lag, but double this concentration is lethal, and concentrations of 1 in 20,000 and less are devoid of appreciable effect.

Methylene blue, safranine, and janus green are much more toxic for infusoria when dissolved in distilled water buffered with $NaHCO_3$ than they are when dissolved in hay infusion which has been similarly buffered.

Concentrations of acriflavine in excess of 1 in 8,000 in hay infusion are lethal, while concentrations above 1 in 20,000 up to 1 in 8,000 are not immediately lethal, but organisms inhabiting these cultures slowly diminish in number. Concentrations lying between 1 in 20,000 and 1 in 200,000 prolong lag indefinitely if the organisms are transferred directly from a seven-day-old parent culture to the culture fluid containing acriflavine. If, however, the organisms are first acclimatized to fresh normal hay infusion by mixing the parent culture with twice its volume of fresh hay infusion from one to five hours before transferring the inocula to the culture fluids containing acriflavine, then lag, although greatly prolonged, is no longer of indefinite duration, and multiplication of the cells ultimately occurs. The succeeding interdivisional period is also greatly prolonged, but if at or shortly before the occurrence of the first division in acriflavine cultures the organisms are transferred again to fresh normal hay infusion, the succeeding interdivisional period is not prolonged, and multiplication proceeds at a normal rate. Furthermore, the capacity of acriflavine to prolong lag and delay multiplication is greater the greater the age of the parent culture from which the inocula are derived. Concentrations lying below 1 in 200,000 in hay infusion delay multiplication, but do not prolong lag. Concentrations of 1 in 2,000,000 and less have no appreciable effect upon lag or reproductive rate.

Acriflavine dissolved in distilled water buffered with $NaHCO_3$ is much more toxic for infusoria than it is when dissolved in hay infusion which is similarly buffered. A concentration of 1 in 10,000 is immediately lethal, and concentrations as low as 1 in 50,000 are slowly lethal for organisms derived from seven-day-old parent cultures. Organisms derived from younger parent cultures are more resistant.

The action of proflavine is similar to that of acriflavine, but low concentrations have less effect than the corresponding concentrations of acriflavine, while

high concentrations are more toxic, so that the action of proflavine in retarding interdivisional processes is exerted over a narrower range of concentrations than that of acriflavine.

Proflavine, dissolved in distilled water buffered with $NaHCO_3$, is about eight times as toxic for infusoria as acriflavine, but for mice acriflavine is from two and a half to five times as toxic as proflavine.

## DISCUSSION OF THE RESULTS.

Setting aside the results obtained with other dyes, which were chiefly negative in character, and confining our attention to the results obtained with acriflavine and proflavine, it is evident that these dyes inhibit the multiplication of infusoria in such a manner as to suggest a specific retardation of some process which is essential to reproduction, for, over an exceedingly wide range of concentrations, none of which exert any other toxic effect which can be recognized as such, the duration of lag is very greatly prolonged, and, if divisions occur at all, the subsequent interdivisional periods are similarly prolonged. Yet division may be induced, in the presence of acriflavine and after a period of lag extending over some days, by the simple procedure of previously exposing the organisms forming the inoculum to their own culture medium suitably diluted with fresh hay infusion, and if such dividing individuals, or individuals which are about to divide, are now transferred to fresh hay infusion, they multiply at a normal rate, displaying no sign of injury consequent upon their prolonged inhabitation of the culture fluid which contained acriflavine.

Retardation of multiplication might conceivably originate in either of two ways, namely, by removal or reduction of a substrate which enters into the syntheses which underlie growth, or by removal or reduction of a catalyser which facilitiates these syntheses. Thus arrest of protein-synthesis, for example, might be due to the lack of availability of a particular amino-acid, or it might be due to destruction or neutralization of a proteolytic enzyme. In the former case growth could not occur at all, unless the missing substrate were furnished in such abundance as to remove the origin of the deficiency by saturating the substance (i.e., acriflavine) which was responsible for its removal. But if the retardation of multiplication were due to destruction or neutralization of an enzyme, then multiplication might be excessively delayed, but still remain possible after sufficient lapse of time, for catalysers do not, as a rule, initiate the reactions which they accelerate. We might, therefore, in such a case find that multiplication of the cells was *slowed*, but not absolutely *prevented*.

The facts obviously favour the latter interpretation, for although the period of lag is almost indefinitely prolonged in 1 in 100,000 acriflavine, in lower concentrations lag and the subsequent interdivisional periods are prolonged, but not indefinitely. Moreover, acclimatization of the organisms by previous exposure

to their own parent culture medium, diluted with fresh hay infusion, permits reproduction to occur, at very long intervals, in concentrations of acriflavine which would otherwise inhibit it altogether.

It is evident, however, that "acclimatization" brings about, directly or indirectly, changes in the organism which are very much more fundamental and enduring than the mere attainment of osmotic equilibrium, which I formerly supposed to be the origin of the effect of this procedure in reducing mortality and lag in subcultures (16).

If the retardation of multiplication which is brought about by acriflavine is due to the removal of a catalyser which facilitates multiplication, then we must also infer that this catalyser is partly protected from the action of acriflavine and released at the moment of cell division. For if, when division is about to occur, organisms are removed from acriflavine cultures into normal hay infusion, reproduction proceeds at a normal rate and no effect of even three days' exposure to acriflavine is observed. If, on the contrary, these organisms are left in contact with acriflavine, the subsequent interdivisional period is very greatly prolonged. This suggests, again, that a part, and possibly the larger part of the catalyst resides in the nucleus in a form which is inaccessible to the acriflavine, and that at cell-division, when the nuclear membrane dissolves, it is liberated and becomes available, in the absence of acriflavine, to facilitate subsequent divisions, or, in the presence of acriflavine, is neutralized and gradually regenerated in the nucleus during the subsequent interdivisional period. We may infer, then, that the catalyser which is removed or inhibited by acriflavine, originates in the nucleus during the interdivisional period, and that at the moment of division it issues into the cytoplasm, and possibly into surrounding medium also. If this be admitted, then it necessarily follows that the proportion which is external to the nucleus, or within the nucleus, and in a condition accessible to the neutralizing action of acriflavine, must contribute to determine the duration of the interdivisional periods, for otherwise lag would not be prolonged, as it is, by acriflavine, and the inhibiting effect of the dye would become evident only after the occurrence of a division.

If this interpretation of the action of acriflavine were correct, then we would expect to find that as the moment of division approaches nearer, so that less synthesis has to be accomplished to induce division, the duration of lag in subcultures containing acriflavine should also diminish, and this, as we have seen, is what actually occurs.

From this point of view, the effect of acclimatization is such as we would expect if the proportion of the catalyser which is accessible to neutralization by acriflavine were reduced by exposure to fresh hay infusion. Equally intelligibly, however, it might be attributable to the removal of products, from the nucleus or from the cytoplasm, or from both, which would otherwise retard the syntheses

underlying multiplication. The organisms, being subsequently removed to fresh culture fluid, would then be removed without these products, which they have lost. Dilution of the parent culture with fresh hay infusion involves increase of tonicity (16), and, as the experiments with phenolphthalein show, decrease of $P_H$ also. Either of these changes in the physical environment might conceivably induce a redistribution of substances between the nucleus and its enveloping cytoplasm, or between the cell and its environment.

In passing, it may be noted that if the action of the acridine dyes upon bacteria, within a higher zone of concentrations, were similar to their effect upon infusoria, we could then interpret the conflicting reports which have been received concerning the antiseptic efficiency of these dyes (18), for we would then expect to find that at certain dilutions the multiplication of the organisms would be checked, or greatly delayed, without accomplishing asepsis. The figures obtained by Dakin and Dunham suggest that this may actually occur (5). Thus, cultures of *Staphylococcus aureus*, in a mixture of blood serum and muscle extract, were rendered aseptic by 1 in 3,000 acriflavine, but in cultures containing 1 in 10,000 acriflavine the numbers of bacteria inhabiting the medium fell, in six hours, to about one-thirtieth of the original number inoculated, but in the succeeding eighteen hours increased until the final population considerably exceeded the original number. Similar phenomena were encountered in the culture containing a similar proportion of proflavine.

## CONCLUSIONS.

1. If acriflavine be added to hay infusion in any proportion between 1 in 20,000 and 1 in 200,000 the period of lag of infusoria (*Enchelys*), inoculated into this medium, is enormously prolonged. Lower concentrations do not prolong lag, but delay subsequent multiplication. Higher concentrations are lethal.

2. Previous "acclimatization" of the organisms by exposure for from one to five hours to the parent culture fluid, diluted by the addition of two parts of fresh hay infusion, permits them to divide, after very prolonged lag, in concentrations of acriflavine which would otherwise inhibit multiplication for an indefinite period. The subsequent interdivisional periods are similarly prolonged, but if, when they are about to divide in a culture containing acriflavine, the organisms are removed to fresh hay infusion the subsequent interdivisional periods are not prolonged and multiplication proceeds, therefore, at a normal rate.

3. The younger the parent culture from which the organisms are derived the less the duration of lag in subcultures containing acriflavine.

4. The action of proflavine is similar to that of acriflavine, but is exerted over a narrower range of concentrations.

5. No other dye investigated, with the exception of janus green, exerted any comparable effect upon the lag or multiplication of infusoria. In a very narrow zone of concentrations in the neighbourhood of 1 in 10,000, janus green causes an indefinite prolongation of lag. Higher concentrations are toxic, lower concentrations without effect.

Incidentally to the main thesis, the following facts were also discovered:

6. Methylene blue, safranine, janus green, and acriflavine are much more toxic for infusoria (*Enchelys*) when dissolved in distilled water buffered with $NaHCO_3$ than they are when dissolved in hay infusion which has been similarly buffered.

7. Scarlet red is soluble to the extent of 0.022 per cent. in five per cent. solutions of saponin.

8. The lethal dose of acriflavine for mice weighing between 20 and 25 grammes is one milligramme. The lethal dose of proflavine is from two and a half to five times as great.

9. When both dyes are dissolved in distilled water buffered with $NaHCO_3$, proflavine is eight times as toxic for infusoria as acriflavine.

The following facts, previously discovered by others (19), have been confirmed:

10. Death, consequent upon administration of acriflavine to mammals, is due to paralysis of the respiratory centre. Doses which are not lethal when administered singly to mammals, may be lethal after two or more successive daily administrations.

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# THE SELECTIVE ABSORPTION OF CHLORINE IONS; AND THE ABSORPTION OF WATER BY THE LEAVES IN THE GENUS *ATRIPLEX*.

by

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In earlier papers, Osborn and Wood (9) and Wood (16) have advanced the view that the arid portions of Australia have developed their own characteristic type of xerophytic flora. This flora, unlike that of other arid regions, has not tended towards succulence, and herein it forms a contrast to the Crassulaceae and Euphorbiaceae in the Karroo, or the Cactaceae in the American deserts. A very considerable degree of xerophily has been attained by some of its members by reducing their transpiration rates to a minimum, owing to peculiar modifications in structure (15) and also by modifying other physiological processes (16).

Rainfall in the drier parts of Australia is extremely variable, and Cannon (3) has pointed out that not all of the actual rainfall is of direct benefit to plants. At some stations the total precipitation may fall in a few showers, whilst in other cases a similar quantity of rain may be distributed over the year in many light showers. From many observations, Cannon, Mussen, and others have concluded that an ecologically effective rainfall in arid and semi-arid regions is one of 0·15 inch or more. But this amount of rain, although effective at one time, i.e., during the rainy period, may not be so at another time when the soil is dry; from which it follows that the time relations of successive storms must be considered. From these considerations it will be seen that a constant minimum supply of ground water is uncertain, but, on the other hand, it is obvious that the successful plants in these regions must be in a state of equilibrium with their environment as regards their water relations.

The characteristic desert shrubs over much of the Central Australian plains within the 10 inch isohyet have been termed tomentose microphylls (Wood, 16). Of these, members of the family Chenopodiaceae are the most prominent, and *Atriplex* and *Kochia* are the most important genera. These plants, particularly those of the former genus, are of great importance to pastoralists, as they afford a fodder reserve in dry seasons. Osborn and Wood (9) tentatively expressed the

view that plants belonging to these genera were able to utilize light falls of rain which would be ineffective so far as absorption by the roots was concerned, since these falls penetrate the soil to a slight extent only. Even after light showers of rain in the drier portions of the year one notices the fresher appearance of the saltbushes, and this led to the further investigation of the possibility of the absorption of water through the leaves. It appeared possible that the peculiar vesicles in *Atriplex* and the hairs in *Kochia* played some part in such absorption.

The work to be described here divides itself into two parts: first, the determination of the absorption of water, and, secondly, the mechanism of such absorption. In connection with the latter, it is necessary to determine the selective absorption of chlorine ions by members of the genus *Atriplex*. Most of the work has been carried out on plants from Koonamore, a sheep station about 250 miles north-east from Adelaide and 60 miles south from Lake Frome. For comparative purposes, however, different plants and soil samples have been collected from widely varying portions of arid and semi-arid South Australia.

## 1. *Water Absorption by the Leaves.*

Difficulties arise in connection with the determination of the amount of water absorbed by the leaves. Spraying the leaves with water has obvious disadvantages, as moisture is liable to be retained in the capillary spaces between the vesicles or hairs which cover the epidermis of the leaves of the tomentose microphylls, and thus cause an apparent absorption. In order to overcome this difficulty, the amount of water absorbed was determined by measuring the increase in weight of a shoot which is placed in an atmosphere near to saturation point with aqueous vapour. In the method finally adopted, the shoot under experimentation was placed in a cylinder totally protected from spray, and the whole placed within a bell jar, which stood over a water seal. Within the jar was an atomizer with a long tube attached, so that the spray could be controlled from outside the jar. By using the spray occasionally a practically saturated atmosphere could be maintained within the jar. The relative humidity inside the jar (as measured with a hair hygrometer) was by this means kept practically constant at 85%. The room temperature, measured by a continuous recording instrument, was 85°F. throughout. All experiments were carried out in a dark room to avoid photosynthesis.

Preliminary trials were carried out on *Atriplex nummularium*. Each shoot was placed alternately inside and outside the jar. The loss of water by transpiration from the shoot outside caused the concentration of salts within the cells to become greater. When these shoots were placed within the jar the amount of water absorbed by the leaves was proportional to the amount already lost, i.e., to the concentration of the cell sap.

| Time. | Inside Jar. *Increase* in grms. per hour per grm. of dry weight of leaves. | | | Outside Jar. *Decrease* in grms. per hour per grm. of dry weight of leaves. | | |
|---|---|---|---|---|---|---|
| | I | II* | III† | I | II | III |
| *1st Day.* Put into jar after cutting from plant. | | | | | | |
| 4 p.m.–10 a.m. | ·0013 | | | | | |
| *2nd Day.* | | | | | | |
| 10 a.m.–4 p.m. | | ·0079 | | ·060 | | |
| 4 p.m.–10 a.m. | ·0057 | | | | ·031 | |
| *3rd Day.* | | | | | | |
| 10 a.m.–4 p.m. | | ·0142 | | ·058 | | |
| 4 p.m.–10 a.m. | ·0072 | | | | ·034 | |
| *4th Day.* | | | | | | |
| 10 a.m.–4 p.m. | | ·0160 | | | | ·160 |
| 4 p.m.–10 a.m. | | | ·012 | | | |
| *5th Day.* | | | | | | |
| 10 a.m.–4 p.m. | | | ·040 | | | |
| 4 p.m.–10 a.m. | | | | | | ‡ |

* Plant allowed to wilt until morning of second day.
† Plant allowed to wilt until morning of fourth day.
‡ Reading not taken. Shoot dry.

In order to render the results comparable, the *increase* in weight of the leaves due to water absorption in the one case, and the *decrease* in weight due to transpiration on the other, are given in grams per hour per gram of dry weight of the leaves under experiment. Absorption of water occurred in every case when the shoots were placed within the jar. This absorption, as the Table 1 shows, was considerable, and the shoots appeared quite fresh after being in the chamber.

After these preliminary trials the investigation was extended and comparative estimations made. The amount of water absorbed was estimated for other species of *Atriplex*. These were *A. vesicarium*, *A. spongiosum*, *A. limbatum*, and *A. velutinellum*. The more important tomentose microphylls associated with the saltbushes were also examined, viz., *Kochia sedifolia*, *K. aphylla*, *Rhagodia spinescens*, and *Bassia obliquicuspis*. All these are members of the Chenopodiaceae. In addition, the amount of water absorbed by plants which have cutinized epidermes was estimated for comparative purposes.

Relative Humidity, 85%. Temperature, 85°F.

Time of experiment in each case, 22 hours: 12 noon to 10 a.m. following day.

| Jar. | No. | Species. | Absorption of water. | Dry weight (grams). | Water absorption per gram dry weight. | Total soluble Salts (% dry weight). | Cl. | NaCl (% dry weight). | Ash % | CaO % | Sulphate $SO_4''$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | 1. | Atriplex spongiosum | +0·610 | 0·820 | +0·744 | 28·0 | 9·2 | 15·2 | — | — | — |
| | 2. | Atriplex vesicarium | +1·185 | 1·150 | +1·102 | 29·6 | 14·3 | 23·5 | — | — | — |
| | 3. | Kochia sedifolia | +0·112 | 2·450 | +0·042 | 6·8* | 3·5 | 5·8 | 24·8 | 18·0 | <0·5 |
| 2. | 4. | Atriplex vesicarium | +1·590 | 1·400 | +1·135 | 29·6 | 14·3 | 23·5 | — | — | — |
| | 5. | Rhagodia spinescens | +0·155 | 0·600 | +0·258 | 8·0* | 2·5 | 4·1 | 28·0 | 20·0 | <0·5 |
| | 6. | Bassia obliquicuspis | +0·660 | 1·200 | +0·550 | 15·0 | 2·1 | 3·5 | — | — | — |
| | 7. | Kochia aphylla | +0·290 | 0·550 | +0·528 | 8·0* | 4·2 | 7·0 | 30·0 | 22·0 | <0·5 |
| 1. | 8. | Atriplex vesicarium | +1·400 | 1·400 | +1·100 | 29·6 | 14·3 | 23·5 | — | — | — |
| | 9. | Eucalyptus corynocalyx | −0·300 | 1·950 | −0·152 | 7·2 | 1·0 | 1·7 | — | — | — |
| | 10. | Sterculia diversifolia | −0·100 | 0·850 | −0·117 | 5·4 | 0·7 | 1·1 | — | — | — |
| | 11. | Acacia decussata | −0·150 | 0·800 | −0·187 | 2·0 | 0·7 | 1·1 | — | — | — |
| 2. | 12. | Atriplex limbatum | +0·540 | 0·600 | +0·900 | 24·0 | 7·1 | 11·7 | — | 10·0 | — |
| | 13. | Atriplex velutinellum | +0·760 | 1·101 | +0·700 | 22·8 | 8·5 | 14·1 | — | — | — |
| | 14. | Kochia sedifolia | +0·155 | 1·702 | +0·090 | 6·8* | 3·5 | 5·8 | 24·8 | 18·0 | <0·5 |

* Soluble salts obtained by deducting CaO from ash.

The results obtained are set out in Table 2. Two jars were used, each with an atomizer. The relative humidity was constant throughout at 85%, and the temperature inside the jar 85°F. The time of experimentation was twenty-two hours in each case. In the sixth column is shown the amount of water absorbed or lost per gram of dry weight of the leaves over this period. It will be seen that in the case of all members of the genus *Atriplex* the absorption is considerable,

ranging from 0·700 grams to 1·135 grams per gram of dry weight of the leaves. The amount absorbed is greatest in *Atriplex vesicarium*.

In order that the results should be strictly comparable, a shoot of *A. vesicarium* was placed in each jar as a control. In each instance the absorption of these shoots was practically constant. With the tomentose microphylls absorption also occurred, but was considerably less in amount. These plants and all species of the genus *Atriplex* have uncutinized, or only thinly cutinized, epidermis. No absorption of water occurred in the case of the cutinized leaves of *Eucalyptus corynocalyx*, *Sterculia diversifolia*, and *Acacia decussata*, but, on the contrary, the leaves lost water by transpiration into the not quite saturated atmosphere.

It is now necessary to account for the much greater absorption of water by the genus *Atriplex* than by the other tomentose plants. To throw light upon the problem an investigation was carried out on the salt content of the leaves from several species of *Atriplex*, special attention being paid to those plants growing in districts with an average annual rainfall of from five to eight inches.

2. *Salt Content and Selective Permeability of the Cells.*

In order to ascertain the salts likely to be present, a detailed analysis of the salts in the leaves of *Atriplex nummularium* was made. This species was selected as it could be obtained in the Botanic Gardens at Adelaide. The result of this analysis follows, the percentages being calculated on the dry weight of the leaves at 105°C.

| | |
|---|---|
| Total soluble salts | 25·00 per cent. |
| Cl . . . . . | 7·78 „ |
| CaO . . . . . | 2·20 „ |
| K . . . . . | 6·20 „ |
| Na . . . . . | 6·00 „ |
| $Fe_2O_3 + Al_2O_3$ . | 0·07 „ |
| Mg . . . . . | Not determined, but present, probably largely from the chlorophyll. |

Oxalic acid is present in the form of calcium oxalate crystals within the cells, and a trace of tartaric acid was also detected. Only a trace of sulphates is present.

It will be seen that the most important salts are chlorides. The leaves analyzed were collected in November, i.e., at the close of the wet season, when, as will be shown below, the salt content is at a minimum; further, *Atriplex nummularium* has a lower percentage of salts than any other species, with the exception of *A. limbatum*. In spite of this fact, salt in these proportions is abnormally great for plants. In such quantities it would be expected to perform some function, and this will be shown to be the case. Many other species of *Atriplex*

from different localities were examined for their chlorine content. At the same time, the soil in which the plants were growing was also collected and analyzed. The results are set out in Table 3.

| No. | Species. | Date. | Locality. | Soil. Air dried at 15°C. Cl. | Soil. Air dried at 15°C. NaCl. | Leaves. Air dried at 105°C. Cl. | Leaves. Air dried at 105°C. NaCl. |
|---|---|---|---|---|---|---|---|
| 1. | Atriplex nummularium | May, 1923 | Botanic Gardens | 0·03 | 0·05 | 10·33 | 17·02 |
| 2. | Atriplex nummularium | Nov., 1923 | Botanic Gardens | 0·03 | 0·05 | 7·80 | 13·00 |
| 3. | A. stipitatum | Mar., 1923 | Port Wakefield | 0·01 | 0·02 | 8·93 | 14·66 |
| 4. | A. stipitatum | May, 1923 | Dilkera | 0·05 | 0·07 | 10·71 | 17·60 |
| 5. | A. stipitatum | Aug., 1923 | Koonamore, N.E. | 0·03 | 0·05 | 7·85 | 13·00 |
| 6. | A. paludosum | Mar., 1923 | Port Wakefield | 0·53 | 0·87 | 13·74 | 22·64 |
| 7. | A. paludosum | Dec., 1922 | Pearson Island | 0·12 | 0·20 | 18·90 | 31·10 |
| 8. | A. paludosum ( ?var.) | Aug., 1923 | Koonamore | 0·03 | 0·05 | 9·28 | 15·29 |
| 9. | A. vesicarium | May, 1923 | Dilkera (open) | 0·04 | 0·06 | 14·20 | 23·50 |
| 10. | A. vesicarium | May, 1923 | Dilkera (with mallee) | 0·03 | 0·05 | 14·20 | 23·50 |
| 11. | A. vesicarium | May, 1923 | Dilkera (with oak) | 0·05 | 0·07 | 14·28 | 23·53 |
| 12. | A. vesicarium | May, 1923 | Dilkera (open) | 0·03 | 0·05 | 13·56 | 22·34 |
| 13. | A. vesicarium | Aug., 1923 | Koonamore (open) | 0·03 | 0·05 | 9·20 | 15·10 |
| 14. | A. vesicarium | Aug., 1923 | Koonamore (with mallee) | 0·03 | 0·05 | 8·57 | 14·10 |
| 15. | A. vesicarium | Aug., 1923 | Koonamore (open) | 0·07 | 0·12 | 9·28 | 15·29 |
| 16. | A. vesicarium ( ?var.) | Aug., 1923 | Koonamore (near lake) | 0·41 | 0·68 | 20·00 | 32·90 |
| 17. | A. vesicarium | Dec., 1924 | Koonamore (near station) | 0·05 | 0·07 | 14·30 | 23·53 |
| 18. | A. vesicarium | Dec., 1924 | Koonamore (near station) | 0·05 | 0·07 | 14·30 | 23·53 |
| 19. | A. vesicarium | Dec., 1924 | Koonamore (near station) | 0·05 | 0·07 | 14·20 | 23·50 |
| 20. | A. halimoides | Aug., 1923 | Koonamore (near station) | 0·07 | 0·12 | 12·49 | 20·58 |
| 21. | A. spongiosum | Aug., 1923 | Koonamore (near station) | 0·01 | 0·02 | 8·92 | 14·70 |
| 22. | A. spongiosum | Dec., 1924 | Koonamore (near station) | 0·03 | 0·05 | 9·20 | 15·20 |
| 23. | A. limbatum | Aug., 1923 | Koonamore (near station) | 0·01 | 0·02 | 5·35 | 8·81 |
| 24. | A. limbatum | Dec., 1924 | Koonamore (near station) | 0·03 | 0·05 | 7·10 | 11·70 |
| 25. | A. velutinellum | Aug., 1923 | Billeroo, N.E. | 0·03 | 0·05 | 14·30 | 23·50 |
| 26. | A. velutinellum | Dec., 1924 | Koonamore | 0·05 | 0·07 | 8·50 | 14·10 |
| 27. | A. angulatum | Aug., 1923 | Koonamore | 0·07 | 0·12 | 12·40 | 20·40 |

On examining these data one sees that the root hairs and also the general tissue cells of *Atriplex* show a high selective permeability towards Cl ions. How such large percentages of chlorides are combined within the cell in order to render them non-injurious to the cell protoplasm is not at present clear. It is possible that loose compounds are formed between the salts and some constituent

of the protoplasm. A preliminary extraction of the proteins from the leaves of *A. vesicarium* with water saturated with ether, according to the method of Chibnall and Schryver (4), and subsequent examination of the residue, showed that a large proportion of globulins was present. Globulins are known to combine with salts.

The amount of salt in the leaves is a function of the amount of salt in the soil, as various examples in the tables show, e.g., *A. vesicarium* and *A. stipitatum*. There is a limit in connection with this, however, as *Atriplex* spp. die in soils containing salt in excess of a certain amount.

In a series of papers Osborn and Wood (9, 10, 11) have shown that the majority of *Atriplex* spp. are not halophytes, as they hitherto have been considered. In the case of *A. paludosum*, at Port Wakefield the plants were dying owing to the high percentage of salt (0·87% NaCl) that had accumulated in the soil at the close of an abnormally dry season.

The salt content also exhibits seasonal variations (vide *A. nummularium*, *A. stipitatum*, *A. paludosum*, *A. vesicarium*, in Table 3). This is due to two factors: first, to the above-mentioned increase in salt content of the leaves simultaneously with increase in salt content of the soil towards the end of a dry season, and, secondly, to the gradual accumulation of salt in the older leaves. This accumulation occurs principally in the vesicles. In the case of plants growing in soils of approximately constant composition, the chloride content of the leaves tends to be constant, during the same season, that is. This feature is shown in *Atriplex vesicarium*, on which somewhat extended research has been made, as it is the commonest saltbush and the one of most importance to the grazing industry. The leaves and soil have been analyzed over two seasons. The salt content of the soil shows only slight fluctuations, but the amount in the leaves shows marked seasonal changes. The first estimation of chlorides was made on plants from Dilkera, in May, 1923, towards the end of a period of five months' drought. The salt content of the leaves estimated as NaCl was 23·5%. In August, 1923, towards the end of the winter, the salt content at Koonamore, was about 15·0%. In December, 1924, after several months' drought, the plants at Koonamore showed 23·5%. In all cases material from several different individuals was used, and these individuals came from widely varying habitats, as shown in Table 3.

*Atriplex* spp. are the only plants which exhibit a high selective absorption of chlorides. The percentage of chloride varies from 12 to 30 per cent. In the other tomentose plants the total chloride content is less than 7 per cent., and in the plants with cutinized leaves the percentage is less than two. The differences between percentages of Cl in soil and leaf are striking in all cases in Table 3.

In the leaves of the *Atriplex* spp. the greater percentage of the soluble salts are chlorides. On examination of the "soluble salts" of the leaves of the tomentose plants, *Kochia*, *Rhagodia*, *Bassia* spp., these "soluble" salts were

found to consist chiefly of calcium oxide. This is due to the method of estimation. The dried leaves were burnt and ignited to convert the large amount of calcium oxalate into oxide, and the residue extracted with boiling water. This was evaporated to dryness and weighed. The "total soluble salts" does not give, therefore, a true value for the salts which exert an osmotic pressure within the leaf. The chloride content, however, does give a basis for comparison. Sulphates are present in an amount less than 0·5% $SO_4$", and are neglected. The chlorine content is given as percentages of NaCl, which is the chief form in which it exists in these leaves. *Kochia* and *Bassia*, which have succulent leaves, resemble true succulents like *Opuntia*, in that the ignited ash consists almost solely of CaO, e.g., *Opuntia macrocentra*: ash = 24·2%; CaO + Mg O = 21·7% (Hilgard, 6).

3. *Osmotic Pressures in the Leaves.*

The high percentages of salt found in the leaves of *Atriplex* exert high osmotic pressures. The osmotic pressures of the sap in the leaves could not be determined directly, as most of the plants were collected in an area 250 miles north-east of Adelaide. Plasmolytic methods are not reliable when dealing with concentrated solutions, and cryoscopic methods cannot be used so far away from civilization. However, an approximate estimate of that portion of the total osmotic pressure which is due to chlorides alone can be obtained. Morse and Frazer (8) have shown that in dilute solutions the osmotic pressure actually measured is identical with the gas pressure predicted from the van 't Hoff equation:

$$P = iRTc$$

Where P is the osmotic pressure, i is Arrhenius' factor, R the gas constant, T the temperature, and c the concentration in moles per litre.

When, however, the solutions become moderately concentrated they found that there was a departure from the linear function between P and c (at constant temperature). The Earl of Berkeley and E. J. G. Hartley (1) have determined the osmotic pressures of concentrated solutions of cane-sugar by measuring the "equilibrium pressure" necessary to prevent osmosis taking place. From their curves an estimate of the pressure exerted by the salts in the leaves can be calculated.

Considering the case of *A. nummularium* (No. 2, Table 2). The leaves of this plant contained 82·04% of water, so that the fresh leaf contains the equivalent of 2·36% NaCl. At this concentration the degree of dissociation of the NaCl molecule is 80%, so that there is present in the cell solution the equivalent of 3·78% undissociated molecules, i.e., 37·8 grams NaCl per litre. This is a ·65M solution of undissociated molecules of NaCl (M = 58·5 grams per litre). A ·65 M solution of cane-sugar, i.e., a solution containing undissociated molecules,

exerts a pressure of about 25 atmospheres, according to Berkeley and Hartley's curves. As was mentioned before, *A. nummularium* contains at the winter stage nearly the minimum percentage of salt found in all the species examined. By calculating the pressure on the lines outlined above for the more typical desert species, *A. paludosum* (No. 7, Table 2) and *A. vesicarium* (Nos. 9, 10, 11, 12), one obtains:

| | |
|---|---|
| *A. paludosum* | 65 atmospheres |
| *A. vesicarium* | 40 atmospheres |

Such extraordinarily high values calculated for the osmotic pressure (calculated in terms of NaCl alone, and disregarding that due to other salts, sugars, etc.) are supported by the work of Harris, Gortner, Hoffman, and Valentine (5), who found a pressure of 67·5 atmospheres in *A. canescens* in the Arizonian deserts, and the extremely high value of 153 atmospheres in *A. confertifolia*. The latter inhabits the salter portions of the desert. This measurement was carried out on leaves collected in July, i.e., during the American summer. The cryoscopic method was used in determining these figures. All these figures are estimated on the expressed sap, but the distribution of the chlorides within the tissues of the leaf of the different species of *Atriplex* is interesting.

4. *Distribution of Chlorides in the Leaf.*

It was pointed out above that the distribution of the osmotic pressures of the cells of the different tissues cannot be determined by the method of plasmolysis owing to the concentration of the solutions to be employed. The amount and distribution of the chlorides themselves were therefore determined. The method adopted consisted in cutting thin sections of the leaves, washing these in distilled water, and then mounting in N/10 silver nitrate solution. The sections were exposed to a 100 candle-power electric light for about half an hour at a distance of nine inches. Under these conditions the silver chloride precipitated by the nitrate formed the coloured sub-salt, and the depth of colour and the amount of precipitation gives the distribution and amount of chloride present in the different tissues of the leaf. It was found desirable after treatment with the silver nitrate and exposure to light to extract the chlorophyll with alcohol. In this way it is easier to observe the sub-chloride.

Before referring to the distribution of chloride it is necessary to describe the structure of the leaf of *Atriplex*. In all the species examined the leaf type is essentially the same as that of *A. vesicarium* (Wood, 15), which is illustrated in the accompanying figure.

The epidermis of the leaf consists of small cells with very slightly cutinized walls. Underneath the epidermis on both sides is a layer of loosely packed cells, containing no chloroplasts, but with clear watery contents. This is the hypodermal layer. The midrib and secondary veins are surrounded throughout their

length by thick-walled, almost cubical cells, with numerous chloroplasts and starch grains. These cells form a complete sheath around the veins. Surrounding these cells is an outer sheath of ordinary thin-walled palisade cells. The epidermis is covered with large bladder-like hairs, which form a scurfy covering to the leaf.

Jung (7), to determine the general distribution of chlorine in plants,

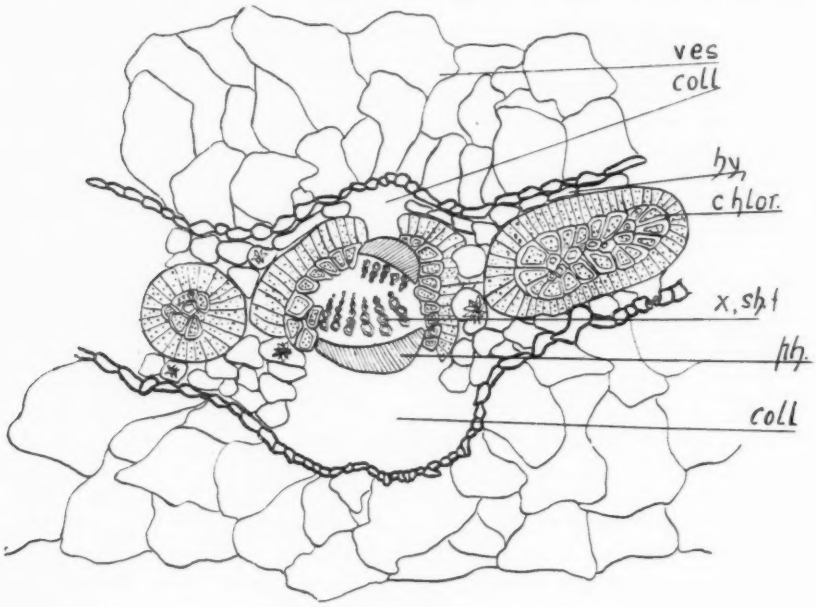


**Figure 1.**

(Reproduced with the permission of the Royal Society of South Australia.)

Transverse section through leaf of *Atriplex vesicarium*.
*ves.*, vesicles; *coll.*, collenchyma; *hyp.*, hypodermal layer; *chlor.*, chlorenchyma; *x.*, *sh.f.*, xylem and sheath fibres; *ph.*, phloem.

Camera lucida outlines, x31.

examined 604 species, embracing 137 families, and found that the chlorides occur in the cell sap, there being a progressive increase in amount from the root upwards. The largest amount was found in the phloem parenchyma, the petiole, and veins of the leaf. Chlorides were absent usually from the mesophyll, the epidermis, and the flower parts. In all the species of *Atriplex* which have been examined, the greatest concentration of salt occurs in the veins of the leaf and in the cubical chlorenchyma surrounding the veins. It occurs in less amount in the epidermis and in the hypodermal cells; there is very little in the thin-walled assimilating cells. In the hairs also there is salt present.

5. *Mechanism of the Absorption.*

The problem now arises as to the mechanism of the absorption of water; it has been shown that *Atriplex* spp. absorb considerable amounts, the tomentose plants less, and the cutinized plants none at all.

Osmotic pressure alone will not account for the absorption. Other pressures may be superimposed upon the osmotic pressure which may increase, decrease, or reverse it. These considerations have been treated by Thoday (13), V. H. Blackman (2), and Stiles (12), and may be summarized as under. The amount of water that enters a plant cell is not, generally, equal to that which should enter hypothetically were the osmotic pressure alone operative in water absorption. At any time, in a cell immersed in water, two pressures are operative. The osmotic pressure P tends to drive water into the cell, and if the cell wall were absolutely elastic water would enter until the concentrations were the same inside and outside the cell. Since the cell wall is not perfectly elastic, there is an inwardly directed pressure exerted by the wall, which tends to force water out of the cell. This pressure is equal and opposite to the turgor pressure T. Therefore, the net pressure sending water into the cell is P—T. Stiles (12) has called this pressure the "suction pressure," instead of "saugkraft," the term originally applied to it by Urspring and Blum (14), since a pressure and not a force is concerned. The suction pressure varies from P, when the cell is non-turgid, to zero when P = T, that is, when equilibrium is reached.

Considering now the absorption of water by the leaves of the plants with their uncutinized epidermis. Wherever there is a water supply available at any point in a tissue which is permeable, water absorption is inevitable, and a gradient of suction pressure will occur, drawing water into the plant, no matter what the initial osmotic pressure may be. When any cell is turgid the suction pressure falls to zero; the neighbouring cells will then take up water, the rate depending on the suction pressure of the neighbouring cells, and assuming no great difference in T within the cells, this will depend upon the osmotic pressure within the cells. This type of absorption has been shown to occur in the first part of the paper in the case of the uncutinized leaves of *Atriplex*, etc., whereas no absorption takes place with the cutinized leaves of *Eucalyptus*, *Sterculia*, and *Acacia*. The point of great interest is the large amount of water absorbed by *Atriplex* spp. This absorption is much greater than that of any of the other tomentose plants; moreover, the saltbushes have an appreciably higher salt content. The more rapid absorption of water in the case of *Atriplex* spp. appears, therefore, to be due to the high osmotic pressures in the tissues of these plants.

This fact is of especial interest, since these saltbushes, and particularly *A. vesicarium*, are the character plants over vast areas of the plains, where they form almost pure communities. Further, their root system is poorly developed: the roots are shallow, being only a few inches beneath the surface; the lateral

roots are deciduous. Extensive absorption of water does not occur at all times through the roots, and the success of the plant in these regions must be attributed to its ability to absorb water rapidly through its leaves.

In conclusion, I wish to thank Professor V. H. Blackman for drawing my attention to the literature on suction pressure; and also Professor T. G. B. Osborn, who collected some of the material and gave helpful suggestions throughout the work.

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# A COLORIMETRIC METHOD FOR THE ESTIMATION OF GUANIDINE AND METHYLGUANIDINE. II. THE DISTINCTION BETWEEN GUANIDINE AND ITS METHYL DERIVATIVES.

by

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In a previous paper (2) the author described a method of estimating guanidine and methylguanidine by means of a reagent which will develop an intense colour in the presence of these substances. It was shown that the colour so produced was directly proportional to the amount of guanidine (or its methyl derivative) in solution, and under certain conditions creatin and creatinine would give no colour with the reagent. If, however, creatin be treated for some time in the presence of excess of the reagent, especially if excess of peroxide of hydrogen be present, it becomes oxidized, liberating methylguanidine, which produces its equivalent of colour. The conversion of creatinine by this means to methylguanidine is a very much slower process, the creatinine becoming firstly hydrolyzed to creatin by the strongly alkaline reagent. However, if the reagent is employed as directed, these reactions do not materially interfere with the accuracy of the determination.

It was noticed early in the experimental work with this reagent that the colour produced by the oxidized creatin would not match that produced by guanidine, the composition of the colour from the liberated methyl derivative being a purplish red, rather than the orange red colour produced by the guanidine standard. A new sample of creatin was prepared and tested with identical results.

This difference, together with other facts, led to the careful examination of the sample of methylguanidine used in the previous experiments (2). Analysis soon led to the discarding of this sample.[1]

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1 The sample was of English origin. It proved to be almost entirely guanidine admixed with a small quantity of methylguanidine. Evidently the methylamine hydrochloride used in its manufacture was highly contaminated with ammonium chloride. This may easily occur if precautions are not taken in the preparation of methylamine.

Another sample of methylguanidine was synthesized after the method of Tatarinow (3) from methylamine and cyanimide. Special precautions were taken in purifying the methylamine used. The cyanimide was obtained from its calcium salt by the method described by Werner (4).

The methylguanidine was separated as the nitrate and recrystallized three times from alcohol. The dried crystals melted at 154°C.

The methyl derivative so produced gave a purplish red colour, which was identical with that produced by the products of oxidized creatin. The colour depth was proportional to the amount of methylguanidine in solution. The colour may be satisfactorily matched with the guanidine standard by interposing an amber coloured glass between the source of light and the colorimeter. The reason for this becomes evident when we examine the absorption spectra (Fig. 1) of the colours.

The guanidine shows a general absorption in the green, blue, and violet, transmitting the red and orange almost completely. The colour thus appears reddish orange.

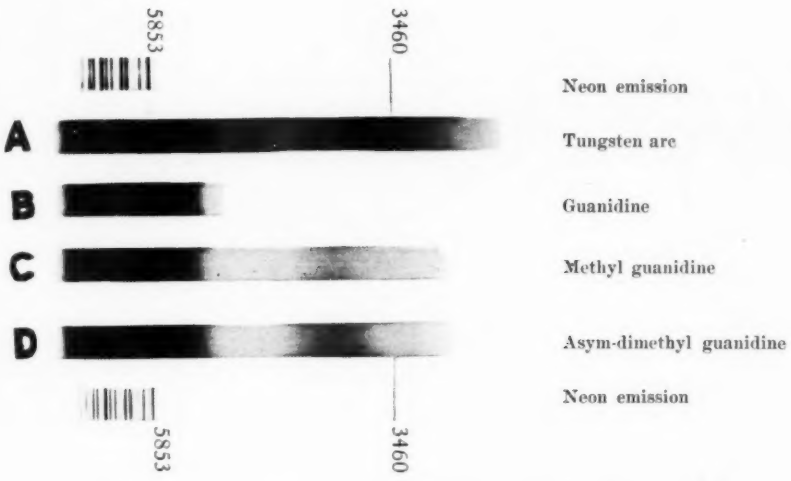
The colour produced by methylguanidine and the asymdimethyl derivative shows a well-marked absorption band in the green and blue, transmitting red, orange, and violet. The colours produced by these two methyl derivatives are identical, and appear a purplish red. If we view the colours in a light devoid of violet, for instance, red or orange, the amount absorbed by equivalent amounts of the two colours becomes the same, and colorimetric comparison of the colour intensities becomes possible.

*Detection in Tissues.*

The estimation of guanidine and methylguanidine in tissues is complicated by the presence therein of substances which inhibit the colour production. Thus uric acid, ammonia, and reducing substances generally, prevent the reaction. These substances must be removed before applying the test. The ammonia may be removed by "permutit", and the uric acid by precipitation with silver lactate (1).

The fact that arginine, creatine, and creatinine, when oxidised by heating for some time with excess of alkaline hydrogen peroxide, will produce methylguanidine, the presence of which may be determined colorimetrically, makes it possible to estimate, by means of the reagent, the amount of guanidyl or methylguanidyl radicles present in biological materials.

I am indebted to Dr. S. W. Pennycuick for his kindness in placing at my disposal the spectrograph employed in obtaining the spectra reproduced in the accompanying figure.




**Absorption spectra of colours produced by guanidine and its methyl derivatives.**

A. Continuous tungsten-arc spectrum. (40 sec. exposure).

B. Same, with 1 centimeter layer of coloured solution, produced by $\frac{\text{mol.}}{400}$ guanidine interposed. (40 sec. exposure).

C. Same as A, with 1 centimeter layer of coloured solution, produced by $\frac{\text{mol.}}{400}$ methyl guanidine interposed. (40 sec. exposure).

D. Same as A, with 2 centimeter layer of coloured solution, produced by $\frac{\text{mol.}}{400}$ asym-dimethyl guanidine interposed. (40 sec. exposure).

The top and bottom are the line spectra of Neon. The wave lengths of the various lines are in Angstrom units.

## SUMMARY.

It is possible to distinguish between guanidine and the methylated guanidines by means of a colour reagent previously described by the author.

Methylguanidine and asym-dimethyl guanidine produce with the reagent a purplish red, whereas the unsubstituted guanidine produces an orange colour.

The intensity of the colours produced is proportional to the quantity of guanidine, or its derivative, present.

The composition and absorption spectra of the colours is discussed.

The conditions requisite for the determination of these substances in tissues are described.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